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Bin1 is a novel tumor suppressor that interacts with and inhibits the oncogenic properties of Myc, which is widely activated in breast cancer. Last year, we generated Bin1 monoclonal antibodies, cloned the human Bin1 gene and promoter, and showed that Bin1 is expressed in normal breast epithelial cells but very frequently missing in malignant breast tumor cell lines. This year, we performed an immunohistochemical study confirming loss of Bin1 in ~50% of a panel of 20 primary breast tumors. Loss of Bin1 was not correlated with other markers tested, supporting utility as a novel and potentially informative marker. Gene deletion did not explain loss of expression. A methylation-specific PCR assay was developed to examine methylation and possible downregulation of the CpG-rich Bin1 promoter. We generated an inducible adenoviral vector system to use for biological tests and showed that Bin1 inhibits growth by apoposis. Deletion analysis identified a key effector domain of Bin1. We cloned the murine Bin1 gene and initiated a 'gene knockout' project. In related work, we showed that Bin1 is required for Myc-mediated apoptosis but also for differentiation of cells containing normal Myc, suggesting why Bin1 may lost so often in various tumors including breast tumors.

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FOREWORD

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Table of Contents

Foreword

Bin1 is a novel tumor suppressor that interacts with and inhibits the oncogenic properties of Myc, which is widely activated in breast cancer. Last year, we generated Bin1 monoclonal antibodies, cloned the human Bin1 gene and promoter, and showed that Bin1 is expressed in normal breast epithelial cells but very frequently missing in malignant breast tumor cell lines. This year, we performed an immunohistochemical study confirming loss of Bin1 in ~50% of a panel of 20 primary breast tumors. Loss of Bin1 was not correlated with other markers tested, supporting utility as a novel and potentially informative marker. Gene deletion did not explain loss of expression. We generated an inducible adenoviral vector system to use for biological tests and showed that Bin1 inhibits growth by apoposis. Deletion analysis identified a key effector domain of Bin1. We cloned the murine Bin1 gene and initiated a 'gene knockout' project. In related work, we showed that Bin1 is required for Myc-mediated apoptosis but for differentiation of cells containing normal Myc, suggesting why Bin1 may lost so often in various tumors including breast tumors.

Introduction

An important goal in breast cancer research is to identify better prognostic tools to predict the course and relapse of malignant carcinoma, and to uncover and develop foundations for the development of novel modalities to treat advanced, intractable disease. Malignant breast carcinomas frequently contain deregulated Myc (Shiu *et al.* 1993). Notably, deregulation is most frequently seen in advanced tumors (due to genetic or epigenetic causes) and, where it occurs, signals poor prognosis (Berns *et al.* 1992; Borg *et al.* 1992; Hehir *et al.* 1993; Watson *et al.* 1993). Supporting the importance of the Myc system in breast cancer, the Myc-regulated genes plasminogen activator inhibitor-1 (PAI-1) (Prendergast and Cole 1989; Prendergast *et al.* 1990) and ornithine decarboxylase (ODC) (Bello-Fernandez *et al.* 1993) are also indicators of poor prognosis (Manni *et al.* 1995; Reilly *et al.* 1992; Sumiyoshi *et al.* 1992). The ability of deregulated Myc to drive apoptosis may provide an Achilles' heel in such cells. Indeed, the ability of the anti-breast cancer drug tamoxifen appears to use Myc-mediated death mechanisms to exert its activity (Kang *et al.* 1996). Therefore, unraveling Myc death mechanisms represent one direction to address a major clinical need.

As part of an effort to learn how deregulated Myc kills cells, we identified the Myc-binding protein Bin1 (formerly called 99). In the background to our proposal we summarized the circumstantial evidence pointing to a role for Bin1 as a breast tumor suppressor gene. In the proposed project, we specifically aimed to:

1. Identify gene mutations and loss of expression in tumor cell lines and primary tumors.
   Tasks 1 and 2 were completed. Tasks 3 and 4 are on schedule and in progress.
2. Ectopically express Bin1 in human tumor and model rodent cell systems and assay its effects on malignant cell growth, cell cycle progression, and apoptotic index.
   Task 5 was completed ahead of schedule. Task 6 was changed because of a technical barrier.: instead of generating inducible cells lines we generated an inducible adenoviral vector. This achievement will still allow us to address Task 7, which will be initiated shortly. With time saved we are expanding the scope of this aim to generate and analyze the breast tumor susceptibility of mice lacking the Bin1 gene.
3. Mutate Bin1 and assay the mutants for growth inhibitory and/or apoptosis activity.
   Task 8 was completed ahead of schedule. Task 9 was initiated and is in progress.

Body

Aim 1. Identify mutations and loss of expression in tumor cell lines and primary tumors

Task 1: Northern and Southern analysis (months 1-12)

Task completed. We have found that Bin1 expression is frequently lost in breast tumor cell lines and primary tumors but not due to gene deletion (data not shown). Since gross gene deletion is not seen, but there is a frequent lack of message, we are testing the hypothesis that aberrant gene methylation leads to epigenetic suppression of gene expression. The Bin1 promoter contains a CpG island (Wechsler-Reya *et al.* 1997b), sites that are methylated and downregulated in cancer cells (e.g. p16INK4 in lung cancer). This year we worked to develop a methylation-specific PCR test which will allow us to examine the ~30 matched pairs of normal and primary breast tumor DNA we have accumulated. The expectation is that

where RNA is expressed we will see fewer signs of methylation than in cases where Bin1 message is absent. Challenges in being able to successfully amplify bisulfite-treated DNA, which is necessary to perform the analysis, slowed us down. We hope to have a test in hand shortly.

Task 2. Immunoprecipitation analysis (months 1-12)

Task completed. We expanded the scope of this Task by performing an immunohistochemical study of 20 cases of frozen normal or malignant breast tissue (see Table I and Figure 1, Appendix for methodology and results). This study indicated that 11/16 tumors had complete or total absence of Bin1 whereas 4/4 cases of normal or benign tissue exhibited Bin1. Losses in tumor cells were not correlated significantly with other markers. This suggests that Bin1 may have a different informative utility as a marker. These findings confirmed frequent loss of Bin1 in breast cancer.

Task 3. Genomic PCR (months 6-36)

Task changed since gene deletion is not the basis for loss of Bin1 expression. Methylation-specific PCR of the Bin1 promoter is instead being performed to assess changed in promoter methylation as the basis for loss of expression (see above).

Task 4: Hybrid mismatch analysis (months 1-48)

Task changed since gene deletion and mutation does not appear to be frequent; instead, Bin1 is downregulated by some epigenetic mechanism.

Aim 2. Ectopically express Bin1 in human tumor and model rodent cell systems and assay its effects on malignant cell growth, cell cycle progression, and apoptotic index.

Tasks 5,6,7. Biological assays.

Task 5 was completed early in the grant period (see last year's report for summary). In Task 6, we generated inducible MCF7 cells lines but these proved to be leaky such that the transgene expression was lost. To circumvent this situation, we developed instead an inducible recombinant Bin1 adenovirus. The gene induction of this virus is based on cre-lox technology. In cells, the Bin1 virus is silent unless cells are coinfected with an Ad-cre virus (see Figure 2). Task 7 was recently initiated on schedule to study the effects of Bin1 induction using this system in a panel of 3 breast cancer cell lines lacking Bin1 (ZR-75-1, T47D, and MDA-MB-468, of which the latter harbors p53 mutation and is estrogen receptor-negative) and the nonmalignant cell line HBL100 which expresses an apparently normal endogenous Bin1 protein. The panel is the same as that used for Western analysis (shown in last year's report). Notably, all tumor cell lines but not HBL100 show a dramatic decrease in viability following ectopic Bin1 expression, accompanied by cell detachment and apoptotic demise within 24 of infection with recombinant adenoviral vector (see Table II and Figure 3). Cytotoxicity is correlated with Bin1, and not with nonspecific effects of the adenovirus or infection, because similar effects were not produced by infection with uninduced vector or with the Ad-cre virus plus empty vector virus (see Table II and Figure 3). Additional results confirming evidence of apoptosis (e.g. TUNEL positivity, membrane blebbing, and DNA degradation) (data not shown). These findings are being confirmed in additional BRCA cell lines where expression of Bin1 has been characterized. Additional evidence for a proapoptotic function for Bin1 in cancer cells has

been documented in cases of prostate carcinoma and melanoma, where Bin1 undergoes loss of function by aberrant splicing (see abstracts of manuscripts in preparation, Appendices).

Given progress we have expanded the scope of this Aim to include generation and analysis of Bin1 'knockout' mice. The goal is to determine whether loss of Bin1 in breast tissue promotes breast tumor progression, either in the absence or presence of activated Myc. This year, we cloned the mouse Bin1 gene (Mao *et al.* 1998) and identified the exons which encode the crucial effector domain (these were defined by structure-function analysis performed as part of Aim 3 (Elliott *et al.* 1998) (see preprints in Appendices). A targeting construct to delete these exons was constructed and heterozygous ES cell clones have been obtained using it that lack one allele of the Bin1 gene (see Figures 4 and 5). We have contracted with a commercial transgenic mouse company to generate chimeric mice which have germ-line transmission of the null Bin1 allele. Current status is blastocyst injection.

In related work, we have established a role for Bin1 in cell differentiation. Tumor suppressors often promote cell cycle exit and/or differentiation in myoblast models (e.g. Rb), so we examined whether Bin1 might have such activities. Consistent with its tumor suppressor qualities, Bin1 was shown by an antisense approach to be necessary for differentiation of C2C12 mouse myoblasts. Overexpression of Bin1 was not sufficient for differentiation, but slowed growth in 10% serum and promoted differentiation once it was triggered (see Wechsler-Reya *et al.* 1998 in Appendices). These observations were validated *in vivo* (see Mao *et al.* 1999 in the Appendices). By examining markers which are elevated at different times during the differentiation program, we established that Bin1 acts at an early point before induction of the earliest marker, the CKI p21WAF1 (see Mao *et al.* 1999 in Appendices). This line of work has dovetailed with a second line of effort and led to the discovery that a fraction of cellular Bin1 also appears in complexes with Rb/E2F in cells (see below).

Aim 3. Mutate Bin1 and assay the mutants for growth inhibitory and/ore apoptotic activity.

Tasks 8,9: Generation and of Bin1 mutants and analysis of their biological activities.

Task 8 to was completed ahead of schedule. Using the Bin1 mutants generated, we defined key effector regions of Bin1 (see preprint of Elliott *et al.* in Appendices). Interestingly, two key regions (termed U1 and Δ4) contain sequence motifs found in the adenovirus E1A protein. We have followed up the idea that Bin1 may act like E1A through its ability to interact with Rb/E2F, a master cell fate regulatory system in cells which is dysregulated in cancer. Using an E2F electrophoretic mobility shift assay to monitor E2F-containing complexes in cells, we have shown that a subset of such complexes contain Bin1 (see Figure 6). The meaning of this biochemical association is not yet clear. How Rb/E2F induce apoptosis is undefined so one idea is that Bin1 may have a role in this process, as it seems to in the case of Myc. Current efforts are to define the exact E2F and Rb members which contact Bin1 (we hypothesize E2F1 and Rb, which are unique in each family for the ability to regulated apoptosis). Bin1 KO cells, when available, will allow us to rule out whether Bin1 has any role in E2F apoptosis.

Conclusions

In year two, we confirmed the frequent loss of Bin1 in a study of primary breast cancers. However, we learned that this loss is not due to gene deletion and promoter methylation is being investigated as the basis. We now have an inducible adenoviral vector in hand to test the biological consequences of

reintroducing Bin1 in breast cancer cells that do not express it. These studies will be complemented in the expanded scope of Aim 2 in which we are generating Bin1 'knockout' mice. Lastly, structure-function studies performed as part of Aim 3 have revealed association of Bin1 with Rb/E2F in cells. This is exciting and suggests that loss of Bin1 could promote deregulation of the malignant properties of E2F as well as Myc.

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Publications related to this grant (see Appendices for preprints and reprints)

1. Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Steller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G.C. (1999). Bin1 functionally interacts with Myc and inhibits cell proliferation through multiple mechanisms. Oncogene, in press.

   • *describes functional assays to be used to analyze the significance of alterations identified in the Bin1 gene in breast cancer cells*

2. Wechsler-Reya, R., Elliott, K., and Prendergast, G.C. (1998). A requirement for the putative tumor suppressor Bin1 in muscle cell differentiation. Mol. Cell. Biol. **18**, 566-575.

   • *confirms a tumor suppressor-like role in promoting cell differentiation*

3. Jensen, D.E., Proctor, M., Ishov, A., Tommerup, N., Vissing, H., Sekido, Y., Minna, J., Borodovsky, A., Wilkinson, K.D., Maul, G.G., Barlev, N., Berger, S.L., Prendergast, G.C., and Rauscher, III, F.J. (1998). BAP1: A novel ubiquitin hydrolase which binds to the BRCA1 ring finger and enhances BRCA1-mediated tumor suppression. Oncogene **16**, 1097-1112.

   • *related collaborative study on a novel BRCA1-binding protein*

4. Mao, N.-C., Steingrimsson, E., Duhadaway, J., Wasserman, W., Ruiz, J., Copeland, N.G., Jenkins, N.A., and Prendergast, G.C. (1999). The murine Bin1 gene functions early in myogenic differentiation and defines a novel region of synteny between human chromosome 2 and mouse chromosome 18. Genomics, in press.

   • *cloning of the mouse Bin1 gene (preliminary study to generate knockout mouse)*

5. Sakamuro, D., Duhadaway, J., Ewert, D., and Prendergast, G.C. A necessary role for Bin1 in c-*myc*-mediated apoptosis. Manuscript submitted.

   • *requirement for Bin1 in Myc-driven apoptosis suggests why Bin1 may be lost in tumor cells*

Abstracts of manuscripts in preparation related to this grant (see Appendices)

1. Elliott, K., Ge, K., and Prendergast, G.C. Caspase-dependent activation of apoptosis in tumor cells by the c-Myc-interacting adaptor protein Bin1.

2. Duhadaway, J., Ge, K., Reynolds, C., and Prendergast, G.C. Frequent loss of expression of the c-Myc-interacting tumor suppressor Bin1 in breast adenocarcinoma.

3. Ge, K., Duhadaway, J., Herlyn, M., Rodeck, U., and Prendergast, G.C. Aberrant splicing of a brain-specific exon causes loss of the tumor suppressor activity of the c-Myc-interacting adaptor protein Bin1 in melanoma.

4. Ge, K., Mao, J., Duhadaway, J., Buccafusca, R., McGarvey, T., Malkowicz, S.B., Tomaszewsky, J.T., and Prendergast, G.C. Alteration of the tumor suppressor Bin1 in prostate adenocarcinoma.

Abstracts presented at meetings related to this grant (see Appendices)

Abstracts describing Bin1 were presented at the following meetings in 1996-97:

a. MD Anderson Breast Cancer Meeting, December 1997, San Antonio TX - poster presentation
b. AAAS Annual Meeting (*Science Innovation* session) - invited oral presentation
c. AACR Annual Meeting, March 1998, New Orleans LA - oral presentation
d. Annual Oncogene Meeting, June 1998, La Jolla CA - poster presentation
e. Cold Spring Harbor Tumor Suppressor Meeting, August 1998, Cold Spring Harbor NY - poster presentation

Personnel supported by this grant

George C. Prendergast, Ph.D.	Principle Investigator
Daitoku Sakamuro, Ph.D.	Research Associate

Appendices

Table I. Frequent loss of Bin1 expression in breast carcinoma. Sixteen cases of malignant breast cancer (including 13 ductal carcinoma, 1 each metaplastic carcinoma, malignant phyllodes tumor, and tubular carcinoma), one case of lactating adenoma, and three cases of benign breast tissue were each subjected to Bin1 immunohistochemistry as described (36). Adjacent sections of each cancer were analyzed for the various pathological parameters and molecular markers as indicated. Lymph node status for each cancer is indicated (LN status; number positive nodes/total nodes examined). ER, estrogen receptor; PR, progesterone receptor; ERB2, c-ERB2 oncoprotein. +, >70% positive cells in section; +/-, 10-70% positive cells in section; +, <10% positive cells in section. N/A, not performed.

Case No.	Diagnosis	Histol Grade	Nuclear Grade	Ploidy	LN status	ER status	PR status	ERB2 status	Bin1 status
1	Infiltr ductal carcinoma	High	High	Aneuploid	10/11	-	-	+	-
2	Infiltr ductal carcinoma	High	High	Aneuploid	0/12	-	-	-	-
3	Infiltr ductal carcinoma	High	High	Aneuploid	1/16	-	-	-	-
4	Infiltr ductal carcinoma	High	Int-high	N/A	0/18	+	+/-	-	-
5	Infiltr ductal carcinoma	N/A	N/A	Diploid	21/21	+	-	N/A	-
6	Infiltr ductal carcinoma	High	High	Aneuploid	8/8	-	-	+	+/-
7	Infiltr ductal carcinoma	High	High	N/A	10/11	N/A	N/A	N/A	+/-
8	Infiltr ductal carcinoma	Int-high	High	Aneuploid	0/16	+	+/-	-	+/-
9	Infiltr ductal carcinoma	High	High	Aneuploid	0/28	-	-	+	+
10	Infiltr ductal carcinoma	Int-high	Int-high	N/A	0/16	N/A	N/A	N/A	+
11	Infiltr ductal carcinoma	High	High	Aneuploid	28/30	+/-	+	+	+
12	Infiltr ductal carcinoma	Int-high	Interm	Diploid	5/13	-	-	-	+
13	Infiltr ductal carcinoma	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+
14	Metaplastic carcinoma	High	High	Aneuploid	5/30	+	+	-	-
15	Malignant phyllodes	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-
16	Tubular carcinoma	Low	Interm	Diploid	N/A	-	-	-	+/-
17	Lactating adenoma	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+/-
18	*Fibrofatty breast tissue*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	+
19	*Benign breast tissue*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	+
20	*Fibrocystic breast tissue*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	+

Summary of Bin1 data:

-: 44% complete loss (<10% positive cells in section; 7/16 tumors)
+/-: 25% partial loss (10-70% positive cells in section; 4/16 tumors)
+: No loss remaining (>70% positive cells in section; 5/16 tumors (31%); 3/3 normal tissues (100%))

=> 11/16 tumors (69%) and 0/4 adenoma or normal tissues exhibited complete or partial loss of Bin1

Table II. Bin1 induces death of malignant breast cells. ~10^5 cells of each of the cell lines indicated with infected with 100 m.o.i. of the viruses indicated. Cells were harvested 48 hr after infection and the proportion of viable cells in the population was determined by trypan blue exclusion. The viable count of cells infected by Adeno-cre and empty adeno vector was set at 100% for comparison (to control for nonspecific effects of the viruses, which reduced cell viability <5% under condition of the assay. The mean of two trials is shown. HBL100 is a nonmalignant breast cell line; the others are malignant and are positive or negative for estrogen-receptor.

Cell line	Adeno-cre + Adeno vector	Adeno-vect + Adeno-MA-Bin1	Adeno-cre + Adeno-MA-Bin1
HBL-100	100	107.8	82.6
MDA-MB-468	100	79.3	33.4
T47-D	100	86.2	45.4
ZR-75-1	100	81.3	29.5

Figure Legends

Figure 1. Examples of results from Bin1 immunohistochemistry in benign and malignant breast tissues. An example of staining patterns are shown for normal breast epithelial from a reduction mammoplasty, a breast benign breast adenoma, and a malignant carcinoma. H&E stain of an adjacent tissue section is shown for comparison below the immunohistochemical slide.

Figure 2. Generation of an inducible Bin1 adenovirus. HepG2 hepatoma cells, a Bin1 null cell line, were infected with mixtures of the Ad-cre recombinase vector and two independent clones (plaque 6 and 15) of the inducible Ad-MA-Bin1 vector. The latter vector contains a ~2 kb stuffer fragment upstream and out-of-frame with a Bin1 expression cassette, which is flanked by loxP sites and excised by cre-induced recombination. The vector is religated after stuffer excision such that the cassette is moved to a position immediately downstream of a strong CMV promoter. Ad-MA-Bin1 was titered against Ad-cre to exhibit induction of Bin1. Cell extracts were prepared 24 hr after infection and subjected to Western analysis with anti-Bin1 antibody 99D (Wechsler-Reya *et al.*, 1997a). Negative control, Ad-cre infection only; Positive control, extract from C2C12 cells which express endogenous Bin1.

Figure 3. Cytotoxic effect of Bin1 in ER$^-$ MDA-MB-468 breast carcinoma cells. Cells were infected with 100 m.o.i. of each of the viruses indicated and photographed 24 hr later.

Figure 4. Targeting construct for homologous recombination with the murine Bin1 gene.

Figure 5. Evidence of homologous recombination in ES cell clones. ES cells were transfected with the targeting construct and G418-resistant cell clones were selected using standard methodology. Genomic DNA was isolated from cell clones and used for PCR using primers which hybridize to a 3' flanking region of the Bin1 gene and a specific segment of the targeting construct. PCR products were subjected to agarose gel electrophoresis, stained, and photographed. Positive signal is consistent with homologous recombination at one allele.

Figure 6. Bin1 is present in some Rb/E2F complexes in cells. C2C12 extracts were subjected to electrophoretic mobility shift assay (EMSA) using a 32P-labelled oligonucleotide derived from the adenovirus E2A gene which contains a strong binding site for E2F proteins. Method was essentially as described by G.C.P. in first studies on the specific DNA binding properties of c-Myc (Prendergast and Ziff, 1991). Preincubation of extracts with ~1 μg of either of two Bin1 monoclonal antibodies which recognize native Bin1 in cell extracts, 99D and 99I (Wechsler-Reya *et al.*, 1997), produced a supershift in the gel. Similar supershifts were not produced by any of 8 other negative control monoclonal antibodies tested as ascites (same as 99D and 99I), one of which is shown in the Figure. The supershift produced by 99D was blocked by preincubation of extracts with the GST-Bin1 immunogen but not unfused GST protein. Western blots of the supershift region of the EMSA gel probed with a polyclonal rabbit antiserum raised to Bin1 (Sakamuro *et al.*, 1996) confirmed the presence of Bin1 in the supershift bands (data not shown).

Meeting abstracts

a. MD Anderson Breast Cancer Meeting, December 1997, San Antonio TX - Poster presentation

Characterization of the putative tumor suppressor BIN1
Daitoku Sakamuro, Katherine Elliott, Robert Wechsler-Reya, and George C. Prendergast
The Wistar Institute, Philadelphia PA 19104

BIN1 was identified through its ability to interact with the functionally critical "box" regions within the MYC N-terminus (1). BIN1 inhibits the oncogenic and transcriptional properties of MYC but also has MYC-independent properties, such as the ability to inhibit cell transformation by the adenovirus E1A and mutant p53 proteins. The structure and function of BIN1 supports a tumor suppressor role in breast and prostate cancer. The BIN1 gene maps to a ≥ 54 kB segment at chromosome 2q14, within a mid-2q region that is a hotspot for deletion in ~40% of metastatic prostate cancers and (at the syntenic locus) in ~90% of murine myeloid leukemias. The BIN1 protein is a short-lived nuclear phosphoprotein that is expressed in most normal cells but that is missing or altered in the majority of breast and prostate carcinoma cell lines and primary tumors examined. Deficits in tumor cells appear functionally significant, because ectopic BIN1 inhibits the growth of cells lacking endogenous expression. Results implicating BIN1 in pathways leading to cell differentiation (in the absence of MYC) or to apoptosis (in the presence of deregulated MYC) will be presented.

1. Sakamuro *et al.* (1996) Nature Genet. 14: 69-77.

b. AAAS Annual Meeting - Invited oral presentation (*Science Innovation* session)

Myc-Bin1 Signaling Pathway in Cell Death and Differentiation. GEORGE C. PRENDERGAST. The Wistar Institute, Philadelphia PA 19104

Myc is a key regulator of cell proliferation that when inappropriately regulated can drive apoptosis. The regulatory mechanism(s) underlying this feature are of interest for their potential utility in the many cancer cells where Myc is deregulated. To gain insight into the mechanism, we previously identified Bin1 as a novel protein that interacts with the crucial N-terminal "Myc box" regions that are hotspots for mutation in cancer cells. Bin1 is a short-lived nuclear phosphoprotein with features of a tumor suppressor that is ubiquitously expressed normally but missing or altered in the majority of tumor cells examined to date. Bin1 associates with Myc *in vivo* and selectively inhibits its transcriptional regulatory properties. Using genetic methods, we examined the function of Bin1 in model systems for cell differentiation and Myc-mediated apoptosis. In C2C12 myoblasts, inhibition of Bin1 prevented the ability of cells to exit the cell cycle and differentiate. Conversely, ectopic Bin1 slowed cell growth and promoted more vigorous differentiation after it was induced. In fibroblasts containing deregulated Myc, inhibition of Bin1 suppressed the ability of Myc to drive apoptosis. Thus, Bin1 may function normally in controlling cell cycle exit and differentiation. However, if Myc is deregulated and cells can not exit the cycle and differentiate fully, Bin1 may generate an abortive apoptosis signal. These findings may explain why Bin1 is so frequently missing in cancer cells, and suggest that its effector pathways may be useful to exploit for therapeutic ends.

c. AACR Annual Meeting, March 1998, New Orleans LA - Oral presentation

Bin1 inhibits Myc target gene expression and is required for cell differentiation and Myc-induced apoptosis. Elliott, K., Sakamuro, D., Wechsler-Reya, R., Basu, A., Staller, P.*, Eilers, M.*, Duhadaway, J., Ewert, D., and Prendergast, G.C. Wistar Institute, Philadelphia PA 19104 USA and *IMT, 35033 Marberg Germany.

Bin1 is a putative tumor suppressor that functionally associates with Myc in vivo. We showed that Bin1 selectively inhibits transactivation of the Myc target genes ornithine decarboxylase (ODC) and prothymosin. Mechanisms involving TBP association and recruitment of an unidentified transcriptional repression activity were implicated. To assess biological function, we inhibited Bin1 by genetic approaches in model systems for myoblast differentiation or Myc-mediated apoptosis. In C2C12 myoblasts induced to differentiate, Bin1 was subjected to alternate splicing and nuclear export. Inhibition of Bin1 prevented cell cycle exit and differentiation and ectopic Bin1 slowed cell growth and promoted a more vigorous response once differentiation was induced. In chick fibroblasts containing deregulated Myc, antisense or dominant inhibitory Bin1 genes reduced the efficiency of Myc-mediated apoptosis. Thus, if Myc is normally regulated, Bin1 is needed for differentiation, whereas if Myc is deregulated and cells can not differentiate fully, Bin1 may generate an abortive apoptosis signal. These findings may explain the reason that Bin1 is frequently missing in many types of carcinoma.

d. Annual Oncogene Meeting, June 1998, La Jolla CA - Poster presentation

Critical Domains and Apoptotic Properties of Bin1, a Putative Tumor Suppressor
Katherine Elliott and George C. Prendergast, The Wistar Institute, Philadelphia PA 19104

Bin1 is a putative tumor suppressor that was originally cloned as a Myc-interacting protein, but it also inhibits cell growth by Myc-independent mechanisms. To begin to distinguish these activities, we defined the regions of Bin1 which are critical for Myc-dependent or Myc-independent growth inhibition in REF cotransformation and HepG2 hepatoma cell growth assays. In addition, we generated a recombinant Bin1 adenovirus to determine how Bin1 suppresses HepG2 growth.

Using a set of deletion mutants, we defined a segment of the Bin1 N-terminal domain (termed BAR-C) that is crucial along with the Myc biding domain (MBD) for suppressing Myc transformation . Both these regions were dispensable for REF transformation by adenovirus E1A or mutant p53, where instead the U1 or SH3 regions of Bin1 were crucial. Interestingly, in HepG2 cells, the MBD was dispensable for growth inhibition but each of the other three domains defined in the REF assay were important. This finding suggest Bin1 may act downstream of Myc and/or that the Myc-independent properties of Bin1 may be to its tumor suppressor functions. A recombinant adenoviral vector was generated to investigate the basis for suppression of HepG2 growth. HepG2 cells infected with adeno-Bin1 exhibited dramatic apoptosis, where infection with a control virus (adeno-lacZ) had no effect. Adeno-Bin1 did not induced apoptosis in normal diploid IMR90 fibroblasts, indicating that Bin1 was not generally toxic to cells. Using the TUNEL assay to detect apoptotic cells, a dose-dependent response was defined with increasing m.o.i. of adeno-Bin1 as compared to adeno-lacZ, with TUNEL-positive cells appearing at 24-36 hr postinfection. Interestingly, a possible G2/M arrest was observed at low m.o.i. of infection and at early time points, suggest that apoptosis may reflect abortive cell cycle arrest. In addition, HepG2 cells stably expressing Bcl-X_L were still susceptible to apoptosis by adeno-Bin1, suggesting that cells rendered resistant to apoptosis by expression of Bcl-2 family proteins are still vulnerable to Bin1. We concluded that the growth inhibitory properties of Bin1 in tumor cells was explained by activation of apoptosis and the BAR-C, U1, and SH3 regions of Bin1 were crucial for this activity.

e. Cold Spring Harbor Tumor Suppressor Meeting, August 1998, Cold Spring Harbor NY

BIN1 STRUCTURE AND EXPRESSION IS FREQUENTLY ABERRANT IN BREAST AND PROSTATE CARCINOMAS AND IN MELANOMA

Daitoku Sakamuro, James B. Duhadaway, Roberto Buccafusca, Nien-Chen Mao and George C. Prendergast. The Wistar Institute, 3601 Spruce Street, Philadelphia PA 19104

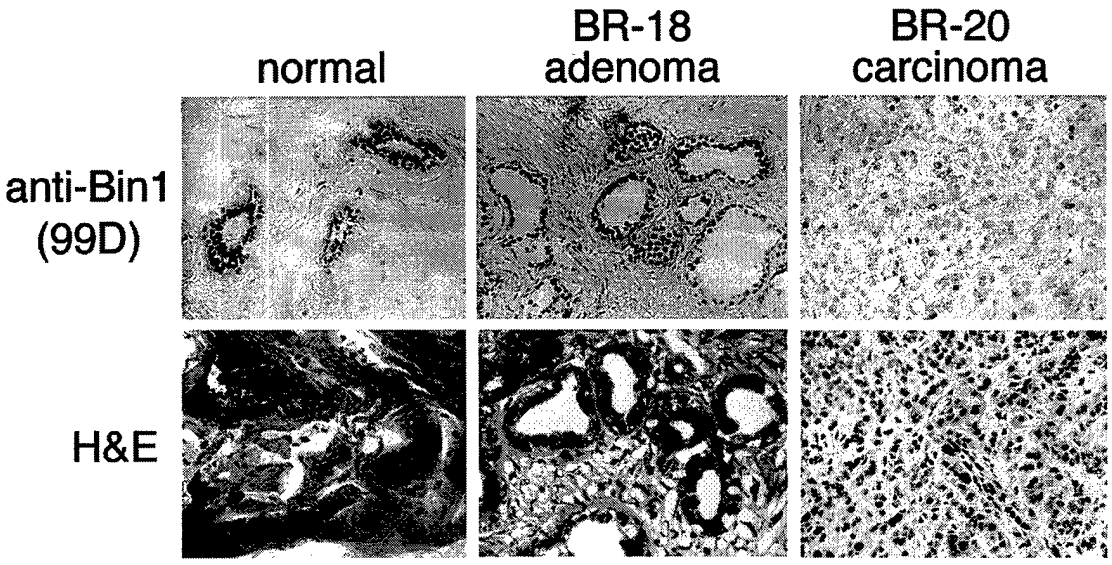
BIN1 is a candidate tumor suppressor that we identified initially through its interaction with the putative transactivation domain of MYC. BIN1 inhibits the transcriptional and oncogenic properties of MYC but it also has MYC-independent growth inhibitory properties, as illustrated by its ability to suppress transformation by adenovirus E1A or mutant p53 (1,2). Recent results suggest that the inhibitory properties of BIN1 are due to apoptosis and that BIN1 may have a necessary role in MYC-mediated apoptosis (3). In previous work, we observed that BIN1 expression was frequently missing in tumor cell lines derived from breast, prostate, liver, and cervical carcinomas. These deficits are important because reintroduction of BIN1 by transfection or adenoviral infection blocks cell growth by apoptosis (1,2,3). A foundation for investigation of BIN1 alteration was provided by the cloning of the human BIN1 gene, which maps to 2q14 within a mid-2q hot spot for deletion in prostate cancer (4,5).

Here we report that BIN1 structure and expression is frequently aberrant in primary breast, prostate, and melanoma tumors and tumor cell lines. Two aberrant patterns of expression and alteration were seen. In breast tumors, frequent loss of expression was observed. BIN1 was undetectable in 5/6 cell lines and 19/28 primary tumors showed complete or partial loss by immunohistochemistry. With the exception of one cell line, we did not observe BIN1 gene deletions by Southern analysis, suggesting that the mechanism for loss was epigenetic. In prostate tumors and melanomas, a second pattern was seen, in which BIN1 levels were frequently elevated in malignant cells and where BIN1 was either aberrantly spliced or mutated. Loss-of-heterozygosity (LOH) at the BIN1 locus was confirmed in the mid-2q region in 6/15 (40%) tumor DNAs examined. Mutational analysis of the remaining allele in these tumors is currently in progress. In prostate tumors, BIN1 was elevated in 29/30 frozen cases examined; the status of the gene is not yet known. In prostate cell lines, BIN1 was normal in androgen-responsive LNCaP but was aberrantly spliced in androgen-independent DU145 and PC3. The alteration involved exon 12A, which is normally only expressed in brain. This type of alteration has also seen in some breast cell lines. Inclusion of exon 12A in BIN1 relieves its inhibitory activity in MYC transformation or tumor cell growth assays. Thus, BIN1 may sustain loss of function in prostate and some breast cancers by aberrent 12A splicing. A similar pattern of overexpression was seen in melanoma, except that structural mutations instead of aberrant splicing was observed. In tumor cell lines, BIN1 was mutated in 6/7 cases; overexpression was seen in 3/6 of those cases as well as in 6/8 metastatic melanoma tumors. Taken together, the results supported the hypothesis that BIN1 is a tumor suppressor which suffers loss of function in tumor cells through a variety of mechanisms, including loss of expression, aberrant splicing, or structural alteration.

REFERENCES:

(1) Nat. Genet. 14, 69-77 [1996]; (2) manuscript submitted; (3) manuscript in preparation; (4) Genomics 33, 329-331 [1996]; (5) J. Biol. Chem. 272, 31453-31458 [1997]

Figure 1



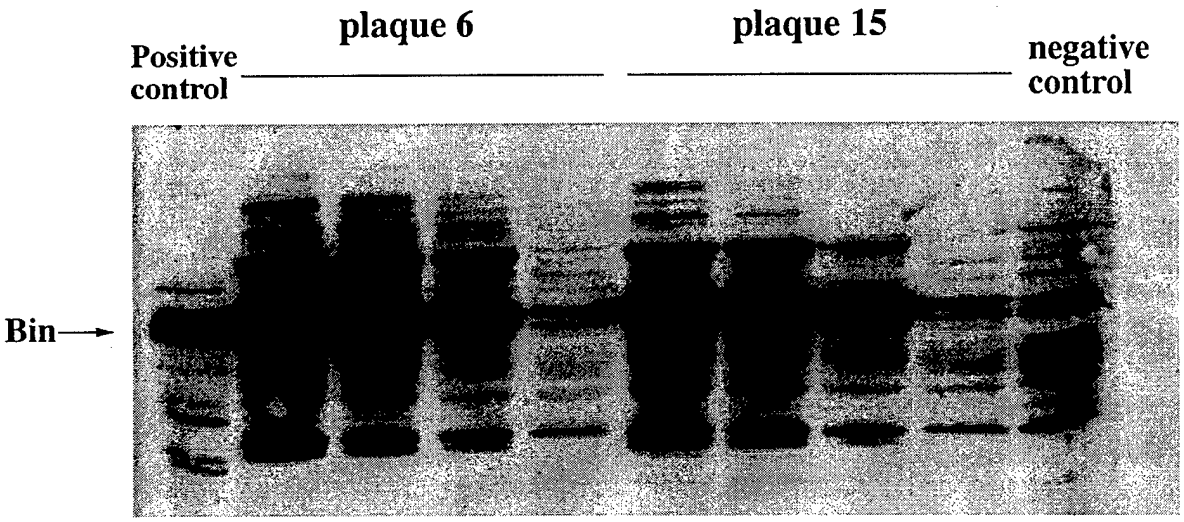


Fig. 2 Western blot of Bin 1 expression

Figure 3

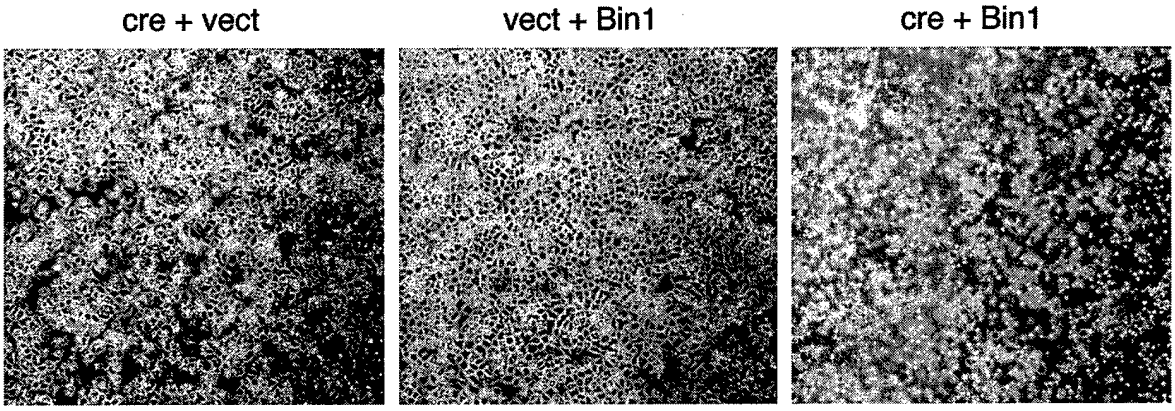


Figure 4

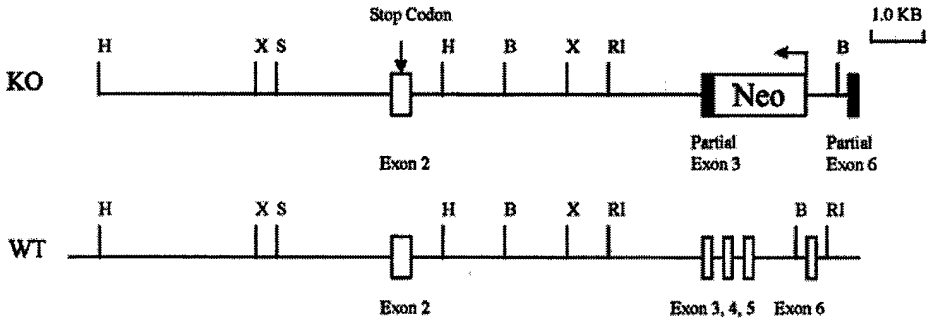

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Figure 5

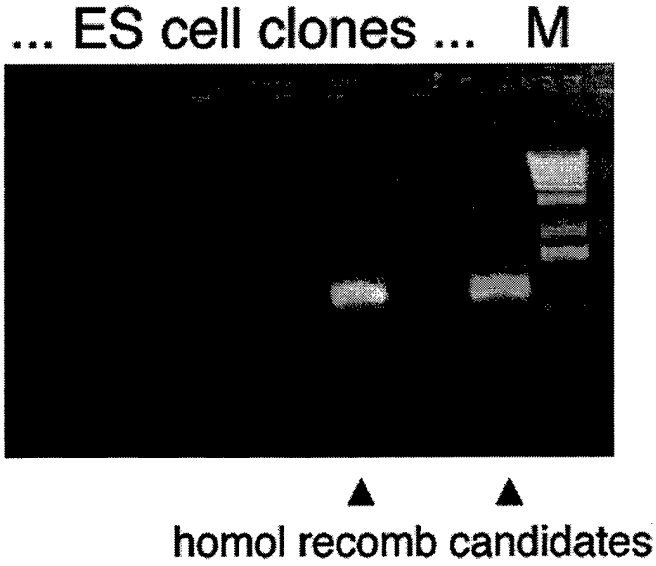
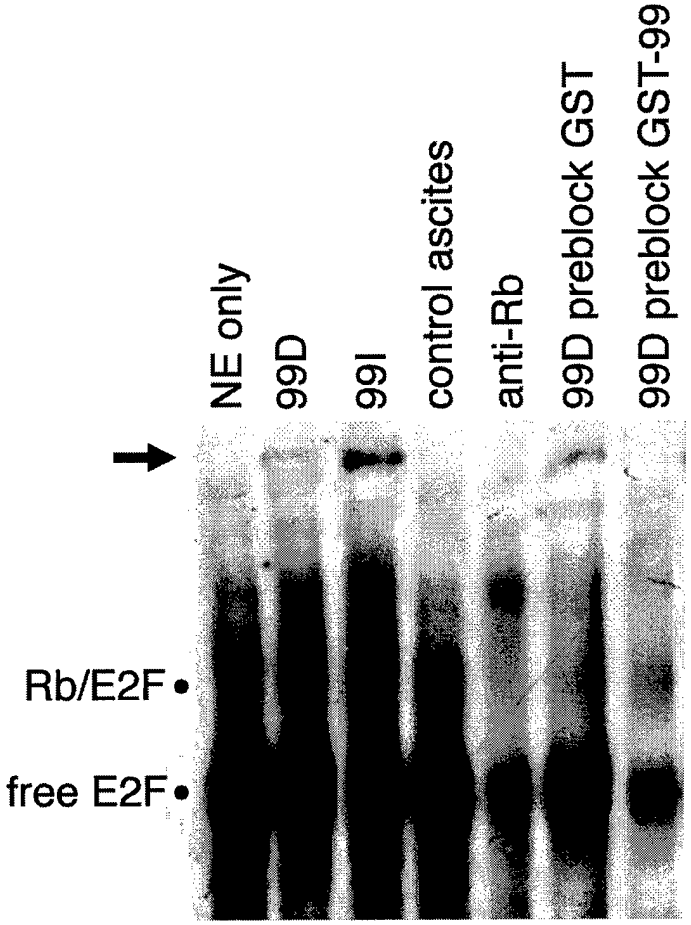


Figure 6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A Role for the Putative Tumor Suppressor Bin1 in Muscle Cell Differentiation

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Bin1 is a Myc-interacting protein with features of a tumor suppressor. The high level of Bin1 expression in skeletal muscle prompted us to investigate its role in muscle differentiation. Significant levels of Bin1 were observed in undifferentiated C2C12 myoblasts, a murine in vitro model system. Induction of differentiation by growth factor withdrawal led to an upregulation of Bin1 mRNA and to the generation of higher-molecular-weight forms of Bin1 protein by alternate splicing. While Bin1 in undifferentiated cells was localized exclusively in the nucleus, differentiation-associated isoforms of Bin1 were found in the cytoplasm as well. To examine the function of Bin1 during differentiation, we generated stable cell lines that express exogenous human Bin1 cDNA in the sense or antisense orientation. Cells overexpressing Bin1 grew more slowly than control cells and differentiated more rapidly when deprived of growth factors. In contrast, C2C12 cells expressing antisense Bin1 showed an impaired ability to undergo differentiation. Taken together, the results indicated that Bin1 expression, structure, and localization are tightly regulated during muscle differentiation and suggested that Bin1 plays a functional role in the differentiation process.

The processes of proliferation, differentiation, and tumorigenesis are intricately related. In normal tissues, immature cells proliferate until environmental signals and intrinsic genetic programs trigger irreversible withdrawal from the cell cycle and terminal differentiation (29, 33). Tumor cells, in contrast, are unable to withdraw from the cell cycle and lack many of the characteristics of differentiated cells (11). This relationship is clinically important, because the degree of dedifferentiation of a tumor cell typically correlates with a poorer prognosis (31). Moreover, interventions that promote differentiation retard tumor growth or even induce tumor regression (7, 9). Thus, proliferation and differentiation are mutually exclusive fates of a cell, and unraveling the mechanisms that control them has clear implications for cancer therapy.

In recent years, many aspects of the genetic programs controlling proliferation and differentiation have been elucidated. In general, these cellular responses are regulated by the opposing actions of two groups of genes, one which promotes cell growth (proto-oncogenes) and the other which opposes it (tumor suppressors) (26). During normal cellular proliferation, growth-promoting genes that control cell cycle entry, DNA synthesis, and cell division are activated by growth factors and by extracellular matrix proteins (4, 32). Inappropriate activation of these genes due to mutation or dysregulation can induce abnormal proliferation and thereby contribute to tumorigenesis (24, 25). During differentiation, many growth-promoting genes (e.g., Myc and cyclin D1) are repressed (36, 43) while many growth-inhibitory genes (e.g., those encoding the retinoblastoma protein and the cyclin-dependent kinase inhibitor p21WAF1) are activated (21, 22). Significantly, differentiation can be inhibited either by forced expression of growth-promoting genes or by inactivation of growth inhibitors (27, 37, 39, 41). Thus, whether a cell grows or differentiates is determined, in large part, by the balance between proto-oncogenes and tumor suppressors.

Bin1 is a novel gene whose features suggest that it may influence this balance (34). Originally identified as a protein that interacts with the N terminus of the Myc oncoprotein, Bin1 is structurally similar to RVS167, a negative regulator of the cell cycle in the yeast *Saccharomyces cerevisiae* (5). Consistent with the notion that it might play a role in regulating cell growth, Bin1 was found to suppress the cell transforming activity of Myc as well as that of the adenovirus E1A and mutant p53 proteins (19, 34). In addition, Bin1 expression is reduced in carcinoma cells derived from malignancies of the breast and other tissues, and introduction of Bin1 into tumor cell lines lacking endogenous expression reduces their proliferative capacity. Finally, the human *Bin1* gene maps to chromosome 2q14 (28), a locus within the mid-2q region that is deleted in >40% of metastatic prostate carcinomas (13). Together, these observations lend strong support to the hypothesis that Bin1 is a tumor suppressor.

Interestingly, analysis of the tissue distribution of Bin1 indicated that the highest levels of expression were in skeletal muscle and brain, tissues which are abundant in postmitotic, terminally differentiated cells (34). Since Bin1 has features of a tumor suppressor, we hypothesized that it might contribute to the regulation of differentiation in these tissues. To investigate this hypothesis, we analyzed Bin1 in an in vitro murine model for muscle differentiation, C2C12 myoblasts (6). In this report, we demonstrate that Bin1 plays a critical role in C2C12 differentiation. After induction of differentiation, Bin1 message and protein levels are dramatically increased and there is a change in the structure of the Bin1 protein due to alternative RNA splicing. This splicing results in a larger form of the protein that localizes in the cytoplasm as well as the nucleus, suggesting a Myc-independent role(s) for Bin1 in differentiated cells. Increased expression appears to be crucial for differentiation, because overexpression of Bin1 promotes myotube formation and upregulation of myosin heavy chain while interference with Bin1 expression significantly impairs these processes.

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MATERIALS AND METHODS

Cell culture. C2C12 cells (kindly provided by David Goldhamer) were carried in growth medium (GM; Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum and penicillin-streptomycin). Cells were grown to approximately 70% confluence and then passaged or induced to differentiate. Differentiation was induced by removing the GM, washing the cells with phosphate-buffered saline (PBS), and then culturing the cells in differentiation medium (DM; Dulbecco's modified Eagle medium supplemented with 5% horse serum and penicillin-streptomycin) for 5 days (or as indicated).

Cells were transfected by using a calcium phosphate precipitation protocol that has been described previously (12). Briefly, 2×10^5 cells seeded in 10-cm-diameter dishes were transfected overnight (18 h) with 15 μg of the appropriate plasmid and 10 μg of pBS+ (Stratagene). The next day, the cells were washed and refed; after an additional 24 h, they were trypsinized and passaged at a 1:25 ratio into new dishes. The following day, G418 was added to 0.8 mg/ml for selection of stable transfectants. The medium was changed every 2 to 3 days, and after 7 to 8 days, individual colonies were ring cloned and expanded into cell lines.

Northern analysis. Total cytoplasmic RNA was isolated from C2C12 cells as described in reference 35. For Northern analysis, 15 μg of RNA was fractionated on an agarose gel and transferred onto a nylon membrane (Duralon-UV; Stratagene). After UV cross-linking, membranes were prehybridized in Church buffer (35) for 4 h at 65°C and then hybridized overnight with a ^{32}P-labeled human Bin1 cDNA probe (generated by random priming) or with an exon 10-specific oligonucleotide probe, 5′-GGAGAATTCGTTGTCACTGTTCTTCTTTCTG (47), labeled by using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals). Membranes were washed twice in 0.1% sodium dodecyl sulfate (SDS)–0.2% saline sodium citrate for 10 min at 50°C and then exposed to film.

Antibodies and blocking proteins. The anti-Bin1 monoclonal antibodies (MAbs) 99D and 99F, generated by immunization of mice with a glutathione *S*-transferase (GST) fusion polypeptide containing amino acids 189 to 398 of human Bin1 (GST-99Pst), are described in detail elsewhere (46). For some immunoprecipitation and Western blotting experiments, 99D was blocked by incubation with a molar excess of a GST fusion polypeptide containing a fragment of murine Bin1 (GST-ATG99) with high affinity for this antibody. Anti-immunoglobulin D (IgD) MAbs (AMS 9.1), used as a negative control for immunoprecipitation and flow cytometry, were a gift from J. Erikson (Wistar Institute). A polyclonal rabbit antiserum to mouse c-Myc (06-213) was obtained from Upstate Biotechnology Inc. MAbs specific for myosin heavy chain (MF20), developed by D. A. Fischman (3), were obtained from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). Fluorescein isothiocyanate (FITC)-coupled goat anti-mouse IgG antiserum, used as a secondary antibody for flow cytometry and immunofluorescence, and horseradish peroxidase (HRP)-coupled goat anti-mouse and anti-rabbit IgGs, used for Western blotting, were obtained from Boehringer Mannheim Biochemicals. For flow cytometry, Western blotting, and immunofluorescence, hybridoma supernatants were diluted 1:20 and secondary antibodies were diluted 1:1,000 (FITC conjugates) or 1:15,000 (HRP conjugates). All antibodies were diluted in PBS plus 0.1% Tween 20 (PBST).

Immunoprecipitation. C2C12 cells were metabolically labeled by incubation for 4 h in methionine- and cysteine-free medium (Life Technologies, Gaithersburg, Md.) containing 100 μCi of [^{35}S]methionine–[^{35}S]cysteine (EXPRESS label; NEN) per ml and then lysed in 1 ml of Nonidet P-40 (NP-40) buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1% NP-40) containing aprotinin, antipain, leupeptin (2 μg/ml each), and phenylmethylsulfonyl fluoride (100 μg/ml). Lysates were spun in a microcentrifuge (Eppendorf) for 15 min at maximum speed to remove insoluble matter, and protein (0.5 mg per sample) was precleared by incubation for 1 h at 4°C with 40 μl of protein G-Sepharose beads. Proteins were immunoprecipitated by incubating lysates for 2 h with 20 μl of protein G-Sepharose beads that had been precoated with 100 μl of hybridoma supernatant (anti-IgD, 99D or 99F plus blocking proteins, added where indicated in the figures). Immunoprecipitates were washed four times in NP-40 buffer, resuspended in 2× SDS-polyacrylamide gel electrophoresis (PAGE) gel loading buffer, boiled for 5 min, and fractionated on a 10% polyacrylamide gel. Labeled proteins were visualized by fluorography.

Western analysis. Cells were lysed in NP-40 buffer, and the lysate was centrifuged to remove insoluble material. Protein (50 μg per sample) was then resuspended in 2× SDS-PAGE gel loading buffer, boiled for 5 min, and fractionated on a 10% polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (Hybond-ECL; Amersham), which were subsequently blocked overnight in PBST containing 5% nonfat dried milk. To detect Bin1 and myosin heavy chain, membranes were incubated for at least 1 h in primary antibody (99D or MF20) and 1 h in secondary antibody (HRP-conjugated goat anti-mouse IgG). To detect c-Myc, membranes were incubated similarly except that anti-c-Myc antibody and HRP-conjugated goat anti-rabbit IgG were used. Membranes were then incubated for 5 min in a chemiluminescent HRP substrate (Pierce) and exposed to film.

Flow cytometry. Proliferating C2C12 cells (10^6 per sample) were trypsinized and washed with PBS. Cells were fixed in PBS containing 0.25% paraformaldehyde for 1 h at 4°C and permeabilized in PBST for 15 min at 37°C. Cells were then stained with primary antibody (99D) for 1 h at 4°C and with secondary antibody (FITC-conjugated goat anti-mouse IgG) for 1 h at 4°C. After being stained, cells were washed three times in PBST and analyzed on a Becton Dickinson FACScan using CellQuest software.

Immunofluorescence. For immunofluorescence analysis, cells were grown on glass coverslips in GM or DM, as indicated in the figures. At the end of the culture period, cells were fixed for 10 min with ice-cold PBS containing 1% paraformaldehyde and then permeabilized for 10 min with ice-cold PBS containing 0.2% Triton X-100. After being washed with PBS, cells were stained for 1 h (at room temperature) with primary antibody (99D or MF20) and for 1 h with secondary antibody (FITC-conjugated goat anti-mouse IgG). Coverslips were washed three times in PBS after each staining step and then mounted on slides with VectaStain mounting medium. Slides were examined and photographed by using a Leitz microscope.

RT-PCR. A murine Bin1 cDNA has been described previously (40). DNA sequences from this cDNA were used to generated the following primers for analysis of the endogenous Bin1 message in C2C12 cells: mNTsen1 (5′-CAGT GCGTCCAGAATTTC) and mNTanti1 (5′-AACACCTTCTGGGCTTTG), mMIDsen1 (5′-AAGCCCAGAAGGTGTTCGAG) and 5′ATG99 (5′-TGGCT GAGATGGGGACTT), and mCTsen1 (5′-CTGAGATCAGAGTGAACCA TG) and mCTanti1 (5′-CACCCGCTCTGTAAAATTC). To detect exogenous Bin1 in transfected cells, the human Bin1-specific primers hX7.1 (5′-GCCAAA ATTGCCAAGGCCGAG) and hAntiNLS2 (5′-GTTGTCACTGTTCTTCTTT CTGC) were used. Reverse transcription-(RT)-PCR was performed as follows. Two micrograms of total cytoplasmic RNA was mixed with 50 pmol of the appropriate primer, heated to 70°C, and cooled rapidly on wet ice. RNA and primers were added to a mixture of Moloney murine leukemia virus reverse transcriptase (Life Technologies) and reaction buffer provided by the manufacturer and incubated at 42°C for 1 h to allow first-strand synthesis. From this reaction mixture, 3 μl was removed and added to a solution containing fresh primers, PCR buffer, and *Taq* polymerase. Samples were subjected to 30 cycles of denaturation (30 s at 94°C), annealing (45 s at 55°C) and polymerization (60 s at 72°C). For each reaction, 10 μl of the product was removed, mixed with sample buffer, and separated on an agarose gel containing ethidium bromide. For further analysis, bands were subcloned into the vector pCR+ (Invitrogen). The DNA sequences of subcloned fragments were determined and analyzed with MacVector and AssemblyLIGN software.

RESULTS

Expression of Bin1 in C2C12 myoblasts. Previous work had indicated that Bin1 mRNA levels in murine skeletal muscle were higher than those in most other tissues (34), suggesting that Bin1 might have a role in this tissue. To begin to address this issue, we examined Bin1 expression in C2C12 cells, a nontransformed myoblast cell line derived from murine skeletal muscle (6). In serum-rich medium, C2C12 cells proliferate rapidly, but when cultured at high density in growth factor-deficient medium, the cells stop dividing, align with one another, express muscle-specific genes, and fuse into multinucleate myotubes (2, 6). Bin1 was immunoprecipitated from extracts of metabolically labeled, proliferating C2C12 cells with 99D, a MAb raised against a human Bin1-GST fusion protein (46). Samples of lysate were also immunoprecipitated with a control antibody (anti-IgD) or with 99D that had been preincubated with a molar excess of nonspecific or specific blocking proteins. Immunoprecipitates were subjected to SDS-PAGE and fluorography (Fig. 1A). 99D specifically recognized a polypeptide of ~65 kDa, similar in size to that generated by in vitro translation of a full-length Bin1 cDNA (34). The ~65 kDa protein from C2C12 cells was not recognized by isotype-matched control antibodies or by 99D that was preincubated with the GST-Bin1 fusion protein (incubation with unfused GST had no effect). We concluded that 99D recognized murine Bin1 in C2C12 cells.

To determine whether Bin1 was expressed throughout the C2C12 population, cells stained with 99D were examined by flow cytometry. A suspension of proliferating cells was generated by trypsinization, then fixed, permeabilized, and stained with 99D or control antibodies followed by fluorescein-conjugated secondary antibodies. Flow cytometric analysis of the stained cell suspension demonstrated that essentially all cells in the population fluoresced above background (Fig. 1B). We concluded that proliferating C2C12 cells universally expressed Bin1 protein.

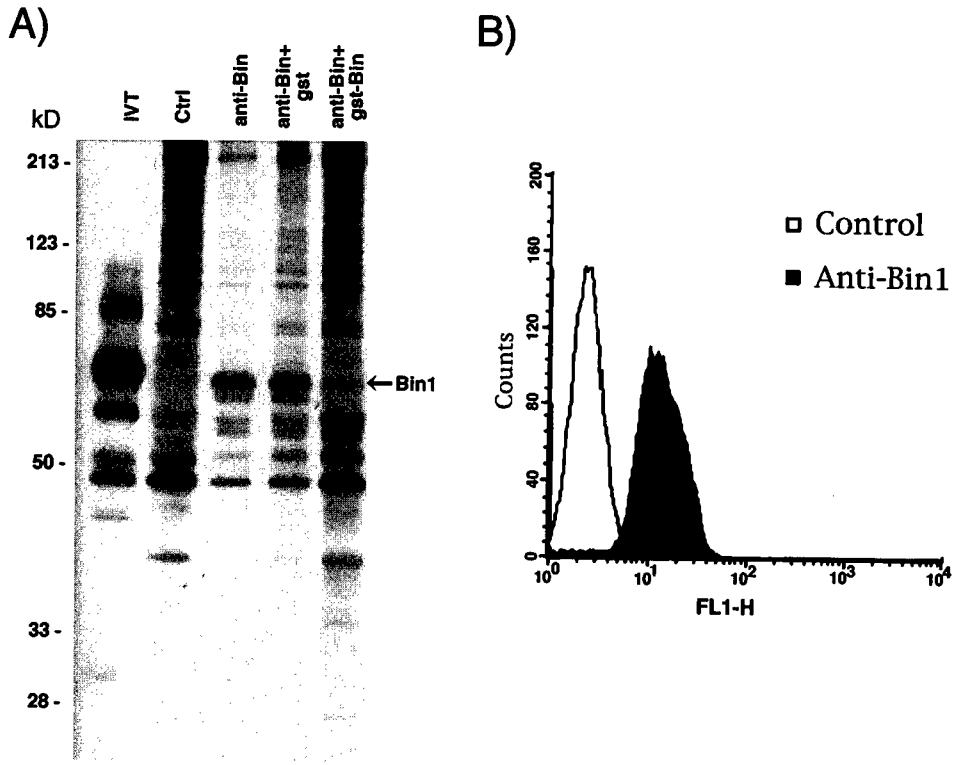

FIG. 1. Expression of Bin1 in C2C12 cells. (A) Immunoprecipitation. C2C12 cells were metabolically labeled with [^{35}S]methionine-[^{35}S]cysteine and lysed in NP-40 lysis buffer. Lysates were precleared and then subjected to immunoprecipitation with anti-IgD antibodies (control [Ctrl]), with 99D (anti-Bin), or with 99D that had been preincubated with GST (anti-Bin+gst) or a protein consisting of GST fused to a murine Bin1 polypeptide (GST-Bin [anti-Bin+gst-Bin]). Immunoprecipitates, along with a ^{35}S-labeled human Bin1 polypeptide generated by in vitro translation (IVT), were analyzed by SDS-PAGE and visualized by fluorography. The positions of molecular mass markers (in kilodaltons) are shown on the left. (B) Fluorescence-activated cell sorter analysis. C2C12 cells were trypsinized to generate a cell suspension and then stained with anti-IgD (control) or 99D (anti-Bin1) antibodies followed by FITC-coupled goat anti-mouse IgG. Cells were then washed and analyzed by flow cytometry. FL1-H, fluorescence channel 1 (FITC).

Bin1 is upregulated during C2C12 differentiation. We next investigated whether Bin1 expression was affected by differentiation. C2C12 cells grown to near confluence and then shifted to DM undergo a pronounced change in morphology; cells elongate, align with one another, and fuse into myotubes (Fig. 2A). In our cultures, morphological differentiation (alignment and fusion) typically began 2 to 3 days following addition of DM, biochemical differentiation (expression of myosin heavy chain; see below) was detectable by days 3 to 4, and myotube generation was maximal (50 to 70% fusion) by day 5 to 6. To assess Bin1 expression during this period, RNA was isolated from cells at various times and subjected to Northern analysis. As shown in Fig. 2B, the level of Bin1 message in C2C12 cells increased dramatically during differentiation (days 1, 3, and 5). Expression began to increase as early as day 2 and reached its highest level at 5 days, when cell differentiation was maximal.

To confirm that the upregulation of Bin1 message was associated with an increase in Bin1 protein, lysates from proliferating or differentiating C2C12 cells were analyzed by Western blotting with 99D (Fig. 3A). Proliferating cells contained a ~65-kDa polypeptide similar to that observed after immunoprecipitation. Following induction of differentiation, the level of this protein increased a few fold. In addition, differentiated cells contained higher-molecular-weight proteins (68 to 70 kDa) that were recognized by 99D. These proteins appeared to be Bin1 related, since they were also observed in immunoprecipitates from differentiated cells (see below) and they were not detected when blots were probed with an isotype-matched control antibody or with 99D that had been preincubated with a GST-Bin1 blocking protein (data not shown).

Since we initially identified Bin1 through its ability to interact with Myc (34), we examined Myc expression in a second population of C2C12 cells that were growing or that had been subjected to serum withdrawal for 2.5 or 5 days. As observed in other cell systems, Myc was rapidly downregulated after induction of differentiation, such that it was undetectable at 2.5 days after serum withdrawal (Fig. 3B). In contrast, it was at this time that one could begin to detect the altered forms of Bin1 that were induced by serum withdrawal. Thus, the increased expression and apparent alteration of Bin1 occurred in cells lacking Myc. We concluded that during C2C12 differentiation, Myc levels decreased whereas Bin1 mRNA and protein levels increased and novel Bin1 species were generated.

Bin1 mRNA is subject to alternate splicing. Although the larger polypeptides that appeared during C2C12 differentiation were immunologically related to Bin1, their structural relationship to Bin1 was not clear. If they represented alternate forms of Bin1, rather than related proteins, the larger and smaller species would be expected to have similar peptide maps. To examine this, 99D immunoprecipitates were fractionated by SDS-PAGE and the larger and smaller species were isolated from gels and subjected to V8 protease mapping. We observed that the different species had virtually identical peptide maps (data not shown), suggesting that they represented different isoforms of Bin1.

Since one explanation for the different sizes of Bin1 was

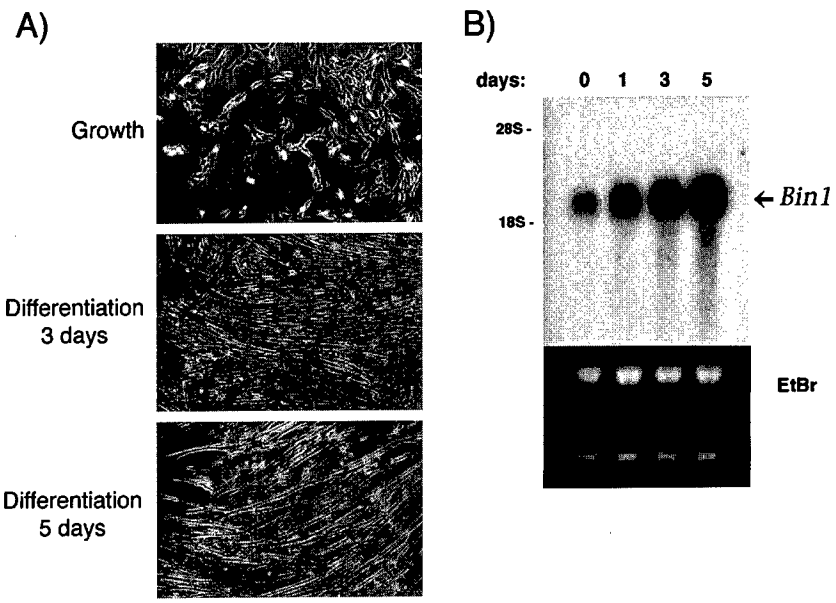

FIG. 2. Bin1 message is upregulated during differentiation. (A) Differentiation of C2C12 cells. Cells were photographed after being cultured in medium containing 15% FCS (Growth) or after 3 or 5 days of culture in medium containing 5% horse serum (Differentiation). (B) Expression of Bin1 mRNA during differentiation. Total cytoplasmic RNA from proliferating (day 0) or differentiating (days 1, 3, and 5) C2C12 cells was separated by agarose gel electrophoresis and blotted onto a nylon membrane. The membrane was probed with a ^{32}P-labeled human Bin1 cDNA and then exposed to film (top panel). RNA integrity and quantity were confirmed by ethidium bromide (EtBr) staining of the gel before transfer (bottom panel).

alternate RNA splicing, we compared Bin1 mRNA structure in proliferating and differentiated cells by RT-PCR. Segments representing the 5′ end, middle, and 3′ end of the Bin1 RNA were amplified with separate sets of primers. The results are shown in Fig. 4A. RT-PCR with the 5′-end primers, corresponding to the N-terminal region of the polypeptide, generated a single band of ~450 bp from RNA from both proliferating and differentiated cells. In contrast, RT-PCR with the midsection primers yielded fragments of ~400 bp from proliferating cells and of 445 bp from differentiated cells. Finally, RT-PCR with the 3′-end primers, corresponding to the C-terminal region of the polypeptide, yielded products of 425 and 515 bp that were present at similar levels in both proliferating and differentiated cells.

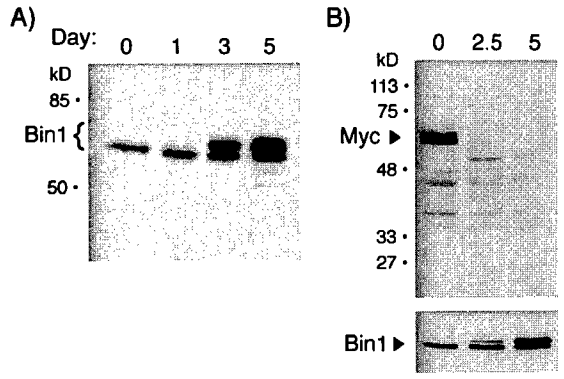

FIG. 3. Induction of novel isoforms of Bin1 and downregulation of Myc during differentiation. (A) Western analysis of Bin1. NP-40 lysates from proliferating (day 0) or differentiating (days 1, 3, and 5) C2C12 cells were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was probed with the anti-Bin1 antibody 99D followed by HRP-conjugated goat anti-mouse IgG. Proteins were detected by chemiluminescence. (B) Western analysis of Myc. Lysates from proliferating (day 0) or differentiating (days 2.5 and 5) cells were analyzed as above, except that a rabbit anti-Myc antibody and HRP-conjugated anti-rabbit IgG were used. The bottom panel shows Bin1 induction on a parallel blot of the same lysates. The positions of molecular mass markers (in kilodaltons) are shown to the left of both panels A and B.

DNA sequence analysis of the 5′ and 3′ RT-PCR products indicated no change in the structures of these regions in proliferating and differentiated cells. The two 3′ products (detected in RNA from both sources) differed in the presence or absence of a 90-bp segment encoding part of the Myc-binding domain (MBD) of Bin1 (19, 34). Significantly, this 90-bp fragment corresponded exactly to an exon conserved in the human gene, exon 13 (47). This result strongly suggested that a murine exon corresponding to human exon 13 was subject to alternate splicing in both proliferating and differentiated C2C12 cells.

A similar analysis of the RT-PCR products amplified with the midsection primers showed that the 400- and 445-bp products found in proliferating and differentiated cells, respectively, were identical except for the presence of an additional 45-bp segment in the latter. This segment is absent from a murine Bin1 cDNA isolated from an embryo library (40) but is present in a human cDNA isolated from a skeletal muscle library (34). As had been observed with the 3′ segment, the 45-bp segment spliced into the midsection was found to correspond to a discrete exon in the human gene, exon 10. Thus, a murine exon corresponding to human exon 10 is alternately spliced into Bin1 mRNA, and this event is regulated during C2C12 differentiation. The splice forms of Bin1 identified in this analysis are summarized in Fig. 4B.

Two additional experiments were performed to verify that exon 10 was expressed only in differentiated C2C12 cells. First, total cytoplasmic RNA from proliferating and differentiated cells was subjected to Northern analysis with an oligonucleotide probe specific for exon 10 sequences. While a full-length cDNA probe recognized Bin1 mRNA from either population, the exon 10-specific probe detected message only in differentiated cells (Fig. 4C). Second, to confirm this difference at the protein level, we used a Bin1 MAb, 99F, that had been deter-

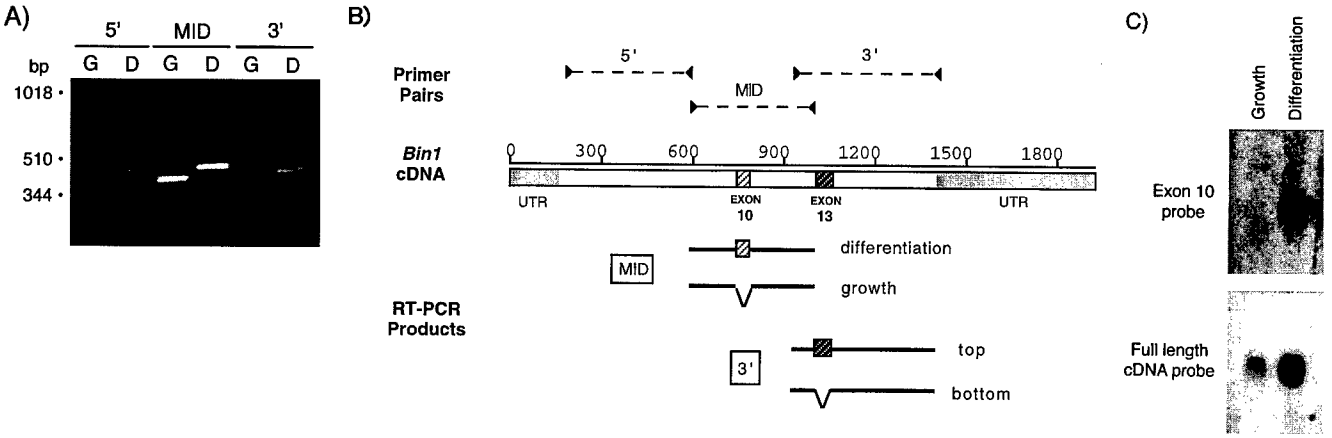

FIG. 4. Differentiation-associated isoforms are generated by alternate splicing. (A) Detection of splicing by RT-PCR. Total cytoplasmic RNA from growing (G) and from differentiated (D) C2C12 cells was reverse transcribed, and the resulting cDNA was amplified by PCR with primers designed to hybridize to the 5′ end, middle (MID), or 3′ end of the murine Bin1 mRNA (see Materials and Methods). PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and photographed. The positions of molecular size markers are shown on the left. (B) Summary of RT-PCR results. The PCR products shown in panel A were sequenced, and the sequences from growing and differentiated cells were compared. 5′ fragments from each population were identical to one another (data not shown). Midregion (MID) fragments from differentiated cells contained a 45-bp sequence (homologous to human exon 10) that was absent in fragments from proliferating cells. Each cell population contained two 3′ fragments; these differed from one another in the presence or absence of a 60-bp sequence homologous to human exon 13. UTR, untranslated region. (C) Detection of alternate splicing by Northern blotting. RNA from growing and from differentiated cells was separated by electrophoresis, transferred onto a nylon membrane, and probed with a ^{32}P-labeled oligonucleotide fragment derived from exon 10 of human Bin1 (top panel) or with a full-length human Bin1 cDNA probe (bottom panel). Membranes were exposed to film for 1 week. No exon 10-positive RNA was detected on film exposed for up to ~3 weeks.

mined to recognize an exon 10-encoded epitope (46). 99F was found to bind in vitro-translated Bin1 polypeptides that included exon 10 sequences but not those that lacked such sequences. Moreover, 99F failed to detect Bin1 protein present in a variety of tumor cell lines, suggesting that the exon 10 epitope was masked or absent in these cells. We employed 99F as a probe to examine the exon 10-containing Bin1 species identified in differentiated C2C12 cells. As shown in Fig. 5, immunoprecipitation of extracts from ^{35}S-labeled C2C12 cells indicated that 99D recognized Bin1 proteins from both proliferating and differentiated cells. In contrast, 99F failed to detect Bin1 in proliferating cells but recognized the larger Bin1 species in differentiated cells. Both the smaller and larger species detected in differentiated cells were heterogeneous. The reason for this was unclear but might reflect differences in phosphorylation states since Bin1 has been shown to be a phosphoprotein (46). We concluded that exon 10 sequences were spliced into Bin1 message during differentiation and that the higher-molecular-weight species of Bin1 protein observed in differentiated cells were due to the expression of exon 10-encoded residues.

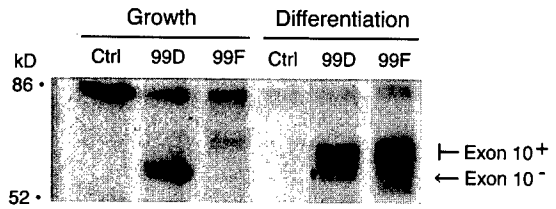

FIG. 5. Differentiation-associated Bin1 proteins can be detected with an exon 10-specific antibody. Growing and differentiated C2C12 cells were metabolically labeled with [^{35}S]methionine-[^{35}S]cysteine, lysed in NP-40 buffer, and subjected to immunoprecipitation with anti-IgD antibodies (Ctrl), 99D, or the exon 10-specific antibody 99F. Immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. The proliferation-associated (Exon 10$^-$) and differentiation-associated (Exon 10$^+$) forms of Bin1 are indicated; note that 99F recognizes only the Exon 10$^+$ form. The positions of molecular mass markers are shown on the left.

Changes in Bin1 structure correlate with changes in cellular localization. To begin to assess the significance of alternate splicing of exon 10 in differentiated cells, we used 99D and 99F to compare the localization of Bin1 in C2C12 cells before and after differentiation (Fig. 6). Consistent with the results described above, in proliferating cells (top panels), Bin1 was detected by 99D but not by 99F. In these cells, as had been observed in other human and rodent cells (34, 46), Bin1 was localized exclusively in the nucleus. In contrast, in differentiated myotubes (bottom panels), Bin1 was detected by 99D as well as 99F, and the pattern of staining with each of these MAbs was distinct. 99D staining was observed in both the nucleus and cytoplasm, while 99F staining appeared predominantly in the cytoplasm, in a fibrous pattern along the length of the myotube. These staining patterns were specific for Bin1, because they were not observed with isotype-matched control antibodies and because they were blocked by preincubation with specific blocking proteins (data not shown). In addition, staining with an antibody specific for myosin heavy chain confirmed that extensive differentiation had taken place in these cultures. Taken together, these results indicated that the low-molecular-weight form of Bin1 in proliferating C2C12 cells was confined to the nucleus whereas the high-molecular-weight, differentiation-associated Bin1 species were found predominantly in the cytoplasm.

Bin1 is necessary for C2C12 differentiation. The complex regulation of Bin1 structure and localization during differentiation suggested that it might play a role in the differentiation process. To test this hypothesis, we investigated the effects of overexpressing sense and antisense forms of human Bin1 cDNA in C2C12 cells. Since alternate splicing of exon 10 (but not exon 13) sequences was tightly associated with differentiation, we also examined the effects of overexpressing a Bin1 species lacking exon 10 sequences to distinguish whether exon 10-encoded information, rather than upregulation of Bin1 expression per se, might be important. Cells were transfected with an expression vector encoding a neomycin resistance gene or the same vector containing a full-length human Bin1 cDNA

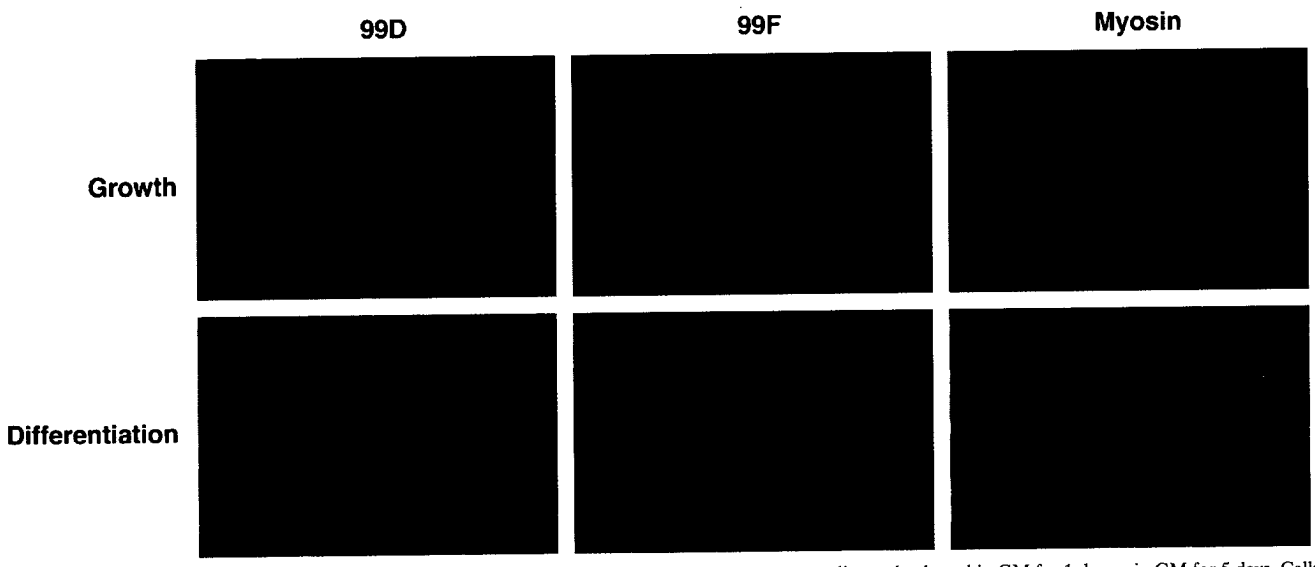

FIG. 6. Localization of Bin1 changes during differentiation. C2C12 cells were plated onto glass coverslips and cultured in GM for 1 day or in GM for 5 days. Cells were then stained with the anti-Bin1 antibody 99D, the exon 10-specific antibody 99F, or the antimyosin antibody MF20, in each case followed by FITC-conjugated goat anti-mouse IgG antibodies. Cells were photographed by using a Leitz microscope.

(sense or antisense). Cell lines derived from G418-resistant colonies were screened for expression of exogenous Bin1 by RT-PCR, using primers specific for the human cDNA that was introduced. To rule out any effects of clonal variation, at least 10 cell lines derived from each vector were generated. A summary of the phenotypes exhibited by each set of cell lines is depicted in Fig. 7.

We observed that sense and antisense lines differentiated better and worse, respectively, than the vector control lines. Only a limited number (10 to 20%) of the cell lines derived from sense cDNA-transfected cells showed elevated expression of Bin1. In addition, cells showing exogenous Bin1 expression grew more slowly than control cell lines, both during and after the selection period (data not shown). These observations argued that Bin1 overexpression might interfere with the growth of C2C12 cells, consistent with results in other cell lines (19, 34). Notably, lines overexpressing Bin1-10 did not show this growth deficit, although they shared with the sense lines a propensity to differentiate more strongly than controls (see below). To further examine the effects of Bin1 overexpression on differentiation, several sense lines were selected for further analysis (from a total of 41 lines generated and phenotypically examined), two of which are reported here (Fig. 8A). Relative to control lines, these cells had significant amounts of exogenous Bin1 mRNA detectable by RT-PCR (top panel). Western analysis of extracts derived from these cells showed two- to fourfold-higher levels of Bin1 protein, as detected with 99D (second panel). Despite the presence of elevated levels of

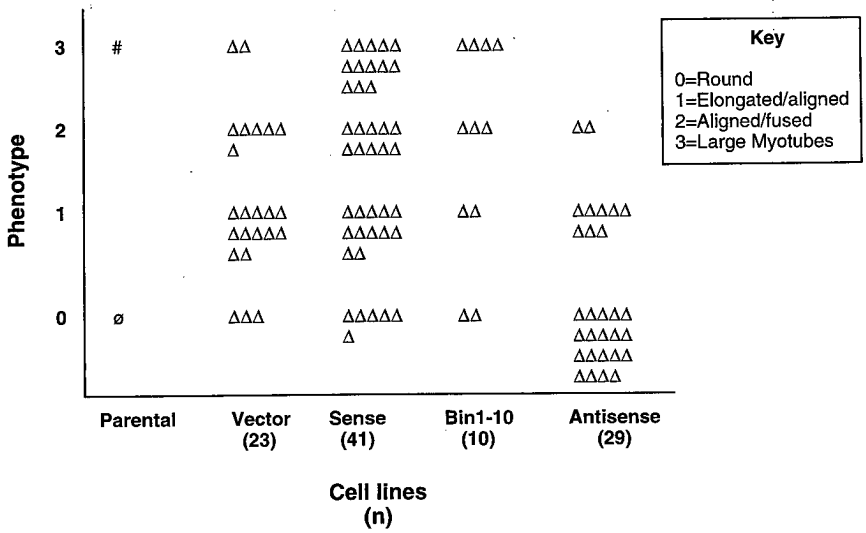

FIG. 7. Phenotypes of C2C12 cell lines. This figure summarizes the phenotypes of clonal cell lines generated by transfection of the indicated vectors. Cells were incubated for 5 to 6 days in DM and then assessed for the phenotypic characteristics noted in the key. Each triangle represents a single cell line. The total number of cell lines examined (n) is indicated beneath the type of vector transfected. The range phenotypes represent clonal variation in the set of cell lines examined; the trend on the y axis represents a greater or lesser tendency toward a differentiated character following incubation in DM. Φ, phenotype of parental cells in GM; #, phenotype of parental cells in DM.

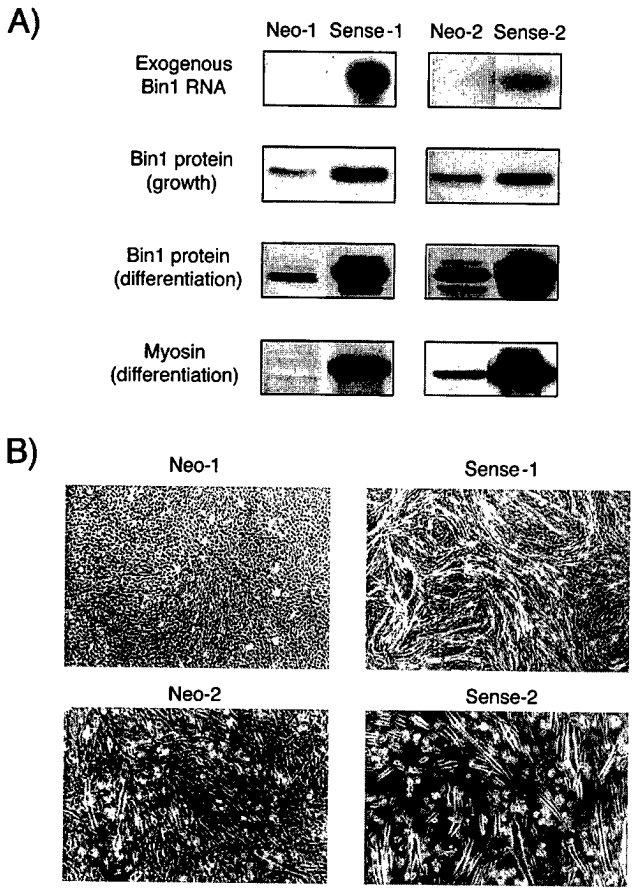

FIG. 8. Overexpression of Bin1 accelerates differentiation. (A) Bin1 and myosin expression. (Top panel) Stable cell lines transfected with empty vector (Neo-1 and Neo-2) or with human Bin1 cDNA (Sense-1 and Sense-2) were selected in antibiotic-containing medium and analyzed by RT-PCR with primers specific for human (exogenous) Bin1. (Second panel) Cells cultured in GM were analyzed for Bin1 protein expression by Western blotting with 99D. (Third panel) Cells were cultured in DM for 3 days, and then Bin1 protein levels were assessed by Western blotting. (The high-molecular-weight forms of Bin1 are presumably generated by alternate splicing of the endogenous mRNA.) (Bottom panel) Differentiated cells were analyzed for myosin heavy chain expression by Western blotting with MF20. (B) Morphology of control and Bin1-overexpressing cells after 3 days in DM. Note the extensive cell fusion in Bin1-overexpressing (Sense-1 and Sense-2) cells compared to controls (Neo-1 and Neo-2).

Bin1, however, their morphology in GM was similar to that of control cells (data not shown), with no evidence of premature alignment or fusion. We concluded that Bin1 overexpression impeded C2C12 proliferation to some extent through a mechanism requiring exon 10-derived sequences but that on its own, Bin1 was insufficient to drive differentiation in GM.

After 3 days in DM, control cells became elongated and aligned but showed limited fusion into myotubes (Fig. 7 and 8B). Consistent with these observations, only modest increases in expression of differentiation-associated isoforms of endogenous Bin1 and myosin heavy chain were observed (Fig. 8A, third and fourth panels). Although control cells showed increased alignment and fusion after longer culturing in DM (see below), they seldom displayed the rate or degree of differentiation observed in parental (nontransfected) cells. This blunted differentiation response in transfected cells might have been due to the high density at which cells were cultured during the drug selection period.

In comparison to control cells, cells overexpressing Bin1 underwent a more rapid and pronounced differentiation in DM (Fig. 7 and 8A). An examination of 12 cell lines overexpressing Bin1 10 showed a similar response trend (data not shown), suggesting that it was the overexpression of Bin1 rather than exon 10-encoded sequences per se that mediated the effect. Notably, cells expressing sense Bin1 or Bin1-10 differentiated even more vigorously than parental C2C12 cells. Within 2 to 3 days of culture in DM, cells exhibited sharp increases in their overall level of Bin1 protein (due to increases in endogenous expression), with significant accumulation of the high-molecular-weight differentiation-associated species (Fig. 8A, third panel). In parallel with this upregulation, there was a dramatic increase in myosin heavy chain levels (Fig. 8A, fourth panel), efficient cell alignment, and extensive cell fusion into myotubes (Fig. 8B). This rapid and efficient differentiation was not vector dependent, because similar phenomena were observed in cells that were transfected with two other Bin1 vectors (data not shown). We concluded that elevation of the levels of Bin1, either containing or lacking exon 10-encoded sequences, was insufficient to induce C2C12 differentiation but accelerated or enhanced the differentiation program once it was initiated.

An examination of antisense cDNA-expressing cell lines suggested that Bin1 was a necessary component of the differentiation program (Fig. 7). Unlike sense transfectants, a significant proportion (50 to 60%) of the G418-resistant cell lines transfected with the antisense vector exhibited expression of the exogenous construct by RT-PCR (Fig. 9A, top panel). Moreover, whereas the sense cDNA-expressing cells were observed to grow more slowly than controls, antisense cDNA-expressing cells proliferated somewhat more rapidly, such that more frequent passaging was necessary to avoid confluence. Several antisense cell lines were selected for further analysis (from a total of 29 lines generated and phenotypically examined), two of which are reported here (Fig. 9A). Western blotting revealed a two- to fourfold decrease in basal levels of Bin1 protein in these cell lines relative to controls (second panel). Similar to sense cDNA-expressing cell lines, the morphology of antisense cDNA-expressing cells in GM was indistinguishable from that of control cells, and these cells did not show an increased tendency to undergo alignment or fusion.

The effects of antisense cDNA expression on differentiation were determined by examining the same set of biochemical and morphological features as before, in cells cultured in DM for up to 6 days (a time point at which control cells exhibited maximal morphological differentiation). Compared to control lines, antisense lines showed significantly less upregulation of differentiation-associated Bin1 species (Fig. 9, third panel). In addition, while control cells exhibited increased levels of myosin heavy chain, antisense cDNA-expressing cells showed little upregulation of this marker (Fig. 9, bottom panel). Finally, while control cells showed substantial alignment and some fusion after 6 days in DM, antisense lines showed little if any alignment, instead retaining the rounded morphology that is characteristic of undifferentiated cells. Taken together with the sense results, these data led us to conclude that upregulation of Bin1 is necessary for differentiation of C2C12 cells.

DISCUSSION

Many genes originally identified through their action in cancer cells have since been shown to play a role in regulating normal cellular growth and differentiation (1, 23, 38). Bin1 was originally identified through its interaction with the N terminus of the Myc oncoprotein (34). Bin1 inhibits the oncogenic and transcriptional properties of Myc but also displays the ability to

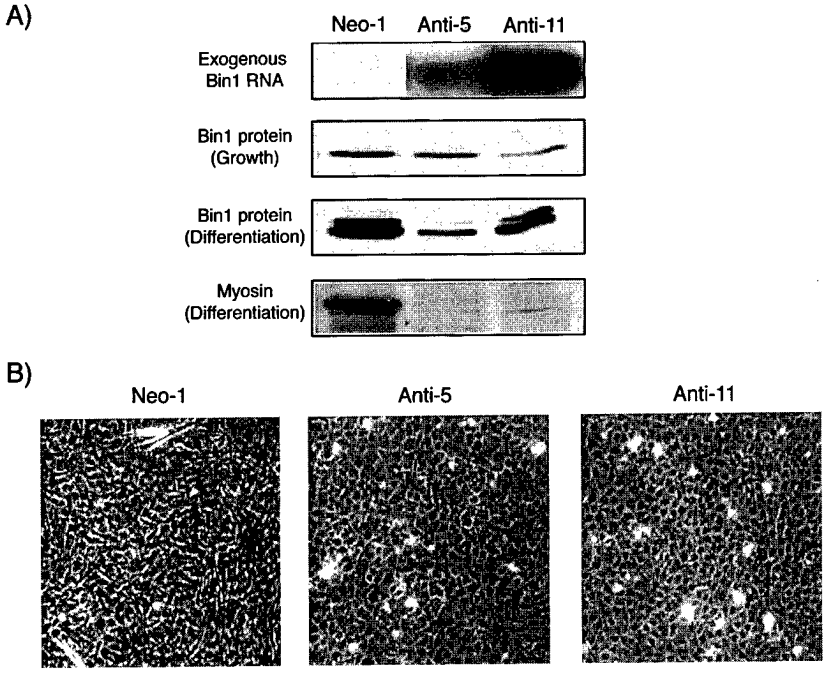

FIG. 9. Antisense Bin1 expression impairs differentiation. (A) Bin1 and myosin expression. (Top panel) Stable cell lines transfected with empty vector (Neo-1) or with antisense Bin1 (Anti-5 and Anti-11) were selected in antibiotic-containing medium and analyzed by RT-PCR for expression of exogenous Bin1. (Second panel) Bin1 protein expression in cells cultured in GM (detected by Western blotting with 99D). (Third panel) Bin1 protein levels in cells cultured in DM for 6 days. (Bottom panel) Myosin heavy chain expression (detected by Western blotting with MF20) in cells cultured in DM for 6 days. (B) Morphology of control (Neo-1) and antisense Bin1 cDNA-expressing (Anti-5 and Anti-11) cells after 6 days in DM. Antisense cDNA-expressing cells appear round and unfused, while control cells show substantial alignment and fusion at this stage.

inhibit cell growth by at least two other Myc-independent mechanisms (19, 34). In this study, we demonstrated that the differentiation of C2C12 myoblasts is accompanied by upregulation and alternate splicing of Bin1 mRNA. This splicing results in the generation of differentiation-specific isoforms of the Bin1 protein which are characterized by their higher molecular weights and distinct patterns of cellular localization. By modulating the amount of Bin1 protein in C2C12 cells, we also demonstrated that Bin1 has an integral role in the muscle differentiation program.

Regulation of Bin1 structure and expression during muscle cell differentiation. Interest in Bin1 in muscle cells was initially stimulated by our observation that murine skeletal muscle expressed higher levels of Bin1 mRNA than most other tissues (34). Consistent with this observation, we found that C2C12 cells contain at least 10-fold-higher levels of Bin1 protein than other cell lines that have been examined. It is noteworthy that these cells express relatively high levels of Bin1 even before myotube differentiation. One possible reason for this comes from studies of the human Bin1 promoter, which have revealed that Bin1 transcription is activated by the myogenic transcription factor myoD (47). Since C2C12 cells are committed to the muscle lineage and already express myoD (1), they may express relatively higher amounts of Bin1 for this reason. Whether Bin1 has a distinct role in the early stages of myogenic commitment in addition to its role in differentiation remains to be determined.

In examining the expression of Bin1 during C2C12 differentiation, we found that Bin1 mRNA levels were dramatically upregulated within 2 days of growth factor withdrawal, at approximately the same time as morphological differentiation was first detectable. Thereafter, Bin1 expression continued to increase as greater numbers of cells aligned and fused into myotubes. In addition to changes in mRNA levels, we observed changes in mRNA splicing during differentiation, with an exon corresponding to human exon 10 (47) being introduced into Bin1 message in differentiated cells. Notably, upregulation and splicing of Bin1 mRNA did not take place when cells were allowed to reach confluence in GM or when growth factors were withdrawn from subconfluent cultures, conditions that do not promote complete morphological or biochemical differentiation (45). Thus, upregulation of Bin1 is intimately linked to activation of a differentiation program.

Several species of Bin1 were found to be generated in C2C12 cells by alternate splicing. Approximately half of Bin1 mRNAs in both proliferating and differentiated cells contained a 3′ sequence corresponding to human exon 13 (47). In differentiated cells, several Bin1 bands were detected by immunoprecipitation and Western blotting, and it is possible that these species differ from one another in expression of exon 13. In proliferating cells, such heterogeneity is not readily apparent; however, longer gels offering higher resolution have revealed closely spaced Bin1 bands that are also consistent with an exon 13 splicing event (45). Interestingly, exon 13 forms part of the MBD of Bin1, which allows it to antagonize Myc-mediated transcriptional activation and cell transformation (34). The fact that exon 13 is subject to alternate splicing suggests that not all Bin1 polypeptides in the cell have Myc-binding capability. Since Bin1 is known to have a Myc-independent as well as a Myc-dependent growth-inhibitory capacity (19, 34), these studies raise the possibility that different functions of Bin1 are mediated by separate species within a cell.

The larger species of Bin1 identified in differentiated cells were shown to result from alternate splicing of a sequence corresponding to human exon 10 (47). While the functional significance of exon 10 splicing remains unclear, its correlation

with cytosolic localization suggests that exon 10 sequences may be responsible for targeting of Bin1 to the cytosol. Counterintuitively, exon 10 encodes a highly basic segment which resembles nuclear localization signal motifs (8, 34). In the context of Bin1, however, this motif is neither necessary nor sufficient for nuclear localization, since Bin1 species that lack exon 10 are found in the nucleus of C2C12 cells, as well as other human and rodent cell lines (46), and species that contain exon 10 are present in the cytoplasm of C2C12-derived myotubes. An alternative function for exon 10 may be revealed by an ongoing analysis of a recently identified Bin1-interacting protein whose binding appears to depend on exon 10-encoded sequences (32a).

Although alternate splicing explains some of the major differences observed in Bin1 species in C2C12 cells, additional complexity of Bin1 structure exists in these and other cells. Work in human cell lines has provided evidence for alternate splicing of another exon in the central region of the Bin1 gene, exon 12A (47), and additional exons are spliced into brain-specific forms of Bin1 (10, 30, 42, 47). While we have not detected any of these exons in mRNA from either human muscle cells or C2C12 cells, they may be relatively rarer and/or spliced at other stages of muscle differentiation or in other cell lineages. Posttranslational modification may also contribute to structural variation, because Bin1 has been found to be phosphorylated in both proliferating and differentiated C2C12 cells (46). In future work, it will be important to analyze the various isoforms of Bin1, since this would provide insights into Bin1 function and into the significance of alternative splicing events in C2C12 and other cell types.

Requirement for Bin1 in muscle cell differentiation. We found that perturbing Bin1 expression in C2C12 cells altered their growth and their susceptibility to induction of differentiation. Expression of exogenous Bin1 (in the sense orientation) interfered with cell growth and promoted cell differentiation. The effects of Bin1 on growth were inferred from the fact that only a small proportion of G418-resistant Bin1 sense cDNA-transfected cells showed overexpression of the exogenous gene by RT-PCR. One interpretation of this finding was that cells expressing high levels of Bin1 had a growth disadvantage and were diluted out during the selection period by cells that expressed lower levels of the protein. Consistent with this notion, the lines that did survive selection expressed only moderate levels of exogenous Bin1 (two- to fourfold-higher levels of expression relative to controls) and grew more slowly than empty-vector control lines. The ability of Bin1 to inhibit cell growth has been documented previously (34), and as noted above, exon 10-encoded sequences may contribute to this property in certain cell lineages, such as muscle cells.

Notably, exogenous Bin1 expression did not promote differentiation of C2C12 cells in GM but dramatically accelerated and enhanced expression of the differentiation program induced by growth factor withdrawal. This accelerated differentiation was observed both morphologically (in terms of cell alignment and fusion) and biochemically (in terms of increased expression of myosin heavy chain and of endogenous Bin1). The fact that Bin1-expressing cells cultured in DM showed more rapid upregulation of differentiation-associated Bin1 isoforms than control or parental cells suggested that Bin1 may positively regulate its own expression, a possibility which needs further investigation.

The analysis of antisense cDNA-expressing cells also strongly supported a role for Bin1 in differentiation. In these cells, the morphological and biochemical features of differentiation were diminished significantly compared to those of control cells. Although we did not determine precisely where Bin1 acts in the differentiation pathway, the facts that Bin1 upregulation occurs relatively quickly (within 2 days of serum withdrawal) and that antisense Bin1 inhibits the earliest morphological signs of differentiation suggest that it may function rather early. Taken together, the data argued that Bin1 upregulation may be a rate-limiting step in the differentiation program.

Although the exact mechanism(s) by which Bin1 acts is unclear, its ability to promote differentiation may reflect both Myc-dependent and Myc-independent activities. Studies of Bin1 structure and function in cell transformation (19, 34) prompt several testable hypotheses. First, as discussed above, Bin1 can interact with the Myc oncoprotein and can inhibit Myc-mediated transcription and transformation. At very early times, before Myc is effectively removed by downregulation, it is possible that Bin1 directly antagonizes Myc's growth-promoting effects and thereby relieves cells of one barrier to differentiation. Previous studies on the role of Myc in muscle differentiation indicate that its overexpression can interfere with biochemical differentiation and/or fusion (15, 16, 27, 29). In this light, Bin1 may directly antagonize the growth-promoting activity of Myc at early times after induction of differentiation, thereby relieving cells of one barrier toward this process. At later times, when Myc is absent, Bin1 would have to act by a Myc-independent mechanism(s). Although we did not define the exact point(s) where Bin1 acts, the altered splicing and relocalization that it undergoes at later times suggests some other role, possibly one affecting cell alignment or fusion. The question of whether Bin1 would be dominant to Myc in C2C12 is somewhat moot because enforced Myc expression is compatible with differentiation (though not cell fusion) in this cell system (15).

In addition to its Myc-related functions, Bin1 also can act in a Myc-independent manner. For example, Bin1 can inhibit transformation of primary rat embryo fibroblasts by the adenovirus gene product E1A, in a manner independent of the MBD (34). Since E1A can inhibit differentiation of myoblasts and reactivate the cell cycle in differentiated myotubes (41, 44), it is possible that Bin1 may counteract these effects as well. In this scenario, Bin1 may function in differentiation by directly or indirectly affecting known targets of E1A, such as the retinoblastoma protein, p107, and p300/CBP (17, 18, 20, 41). Similarly, we have observed that Bin1 can inhibit cell transformation by a dominant inhibitory mutant of p53 (19). Although the mechanism of this effect is not clear, the fact that p53 function is required for C2C12 differentiation (39) raises the possibility that Bin1 also exerts its effects on differentiation via p53. Future analysis of stable C2C12 lines that overexpress Bin1 mutants defective in E1A and/or p53 inhibition may shed light on the pathways involved in BIN action.

Our studies provide strong evidence that Bin1 can regulate muscle differentiation. Since Bin1 is expressed ubiquitously (34), it may also contribute to the control of differentiation programs in other cell types. Consistent with this possibility, we have noted that during induction of the monocytic differentiation program in the promyelocytic cell line HL-60 (14), Bin1 expression and splicing patterns are altered in a manner similar to that observed in C2C12 cells (45). Thus, Bin1 may have a general role in cell differentiation. If so, the frequent loss of Bin1 may contribute to malignant development both via the loss of processes required for terminal differentiation and by contributing to Myc deregulation.

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The Murine *Bin1* Gene Functions Early in Myogenesis and Defines a New Region of Synteny between Mouse Chromosome 18 and Human Chromosome 2

Fn1-2

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We cloned and functionally characterized the murine *Bin1* gene as a first step to investigate its physiological roles in differentiation, apoptosis, and tumorigenesis. The exon–intron organization of the ≥55-kb gene is similar to that of the human gene. Consistent with a role for *Bin1* in apoptosis, the promoter included a functional consensus motif for activation by NF-κB, an important regulator of cell death. A muscle regulatory module defined in the human promoter that includes a consensus recognition site for myoD family proteins was not conserved in the mouse promoter. However, *Bin1* is upregulated in embryonic development by E10.5 in myotomes, the progenitors of skeletal muscle, supporting a role in myogenesis and suggesting that the mouse and human genes may be controlled somewhat differently during development. In C2C12 myoblasts antisense *Bin1* prevents induction of the cell cycle kinase inhibitor p21WAF1, suggesting that it acts at an early time during the muscle differentiation program. Interspecific mouse backcross mapping located the *Bin1* locus between *Mep1b* and *Apc* on chromosome 18. Since the human gene was mapped previously to chromosome 2q14, the location of *Bin1* defines a previously unrecognized region of synteny between human chromosome 2 and mouse chromosome 18. © 1999 Academic Press

INTRODUCTION

The identification and functional analysis of tumor suppressor genes are major goals of cancer research. Bin1 is a tumor suppressor in breast and prostate carcinoma that was identified through its ability to interact with the transcriptional regulatory domain of the Myc oncoprotein (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a). Bin1 inhibits the oncogenic and transcriptional properties of Myc (Sakamuro *et al.*, 1996; Elliott *et al.*, submitted for publication) but it can also inhibit cell growth by Myc-independent mechanisms (Elliott *et al.*, submitted for publication). A necessary role has been defined for Bin1 in differentiation of C2C12 myoblasts (Wechsler-Reya *et al.*, 1998). Recent work has identified Abl as a second oncoprotein that interacts with Bin1, through its SH3 domain (Kadlec and Pendergast, 1997) that is dispensible for Myc interaction (Sakamuro *et al.*, 1996). The terminal regions of Bin1 are structurally similar to amphiphysin, a neuron-specific protein that is a paraneoplastic autoimmune antigen in breast and lung cancer (David *et al.*, 1994; Dropcho, 1996), and to RVS167 and RVS161, two negative regulators of the cell cycle in yeast (Bauer *et al.*, 1993; Crouzet *et al.*, 1991). Amphiphysin has been implicated in receptor-mediated endocytosis (David *et al.*, 1996; Wigge *et al.*, 1997b), and brain-specific splice forms of Bin1, also termed amphiphysin II or amphiphysin-related protein (Butler *et al.*, 1997; Ramjaun *et al.*, 1997; Tsutsui *et al.*, 1997), have been reported to interact with amphiphysin and to influence endocytosis (Wigge *et al.*, 1997a; Owen *et al.*, 1998). Taken together, the results suggest that Bin1 is a nucleocytoplasmic adaptor that participates in a signaling pathway(s) linking membrane trafficking with gene and cell cycle regulatory events.

The human *BIN1* gene has been cloned and characterized (Wechsler-Reya *et al.*, 1997b). It is ubiquitously expressed and extensively alternately spliced with highest expression in skeletal muscle. *BIN1* is located at chromosome 2q14 (Negorev *et al.*, 1996), within a mid-2q region that is deleted in ~42% of metastatic prostate cancers (Cher *et al.*, 1996). We cloned the mouse *Bin1* gene (alternate symbol *Amphl*) as a prerequisite to generating homozygous null animals that

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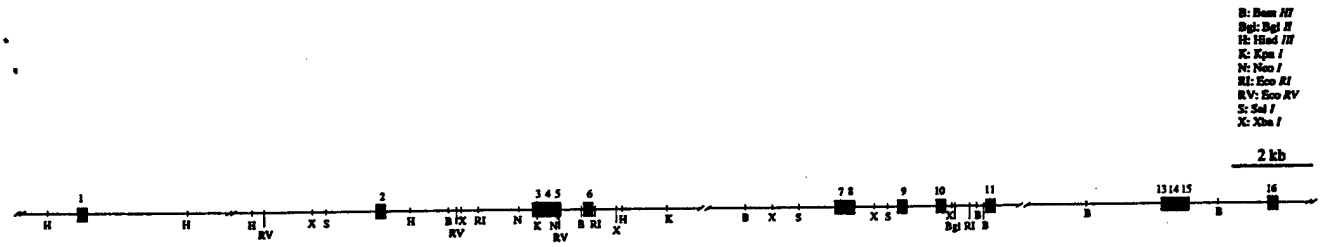

FIG. 1. Physical map of the mouse *Bin1* gene. A restriction map of four contiguous regions of the gene derived from subclones from a single BAC clone is shown. Exons located by Southern analysis with a murine cDNA and limited DNA sequencing were numbered by comparison to the human gene (Wechsler-Reya *et al.*, 1997b).

will allow investigations of its physiological functions. In this study, we defined the exon–intron organization, promoter, and muscle-specific expression of mouse *Bin1*. We also obtained evidence supporting a function for *Bin1* in regulating apoptosis and myogenesis. Finally, we mapped the chromosomal location of the gene to mouse chromosome 18, a locus that defines a new region of synteny with human chromosome 2.

MATERIALS AND METHODS

Gene cloning and characterization. Three clones were isolated from a murine 129/Sv BAC library (Genome Systems) by hybridization with a full-length human cDNA (Sakamuro *et al.*, 1996). Restriction fragments were subcloned into pKS II (−) (Stratagene) and analyzed by extensive restriction mapping and Southern analysis with human Bin1 cDNA probes. The DNA sequences of exon-containing subclones were determined using an automated DNA sequencer. Primer sequences were derived from a full-length murine cDNA, SH3P9 (Sparks *et al.*, 1996), which encodes the ubiquitously expressed splice form of Bin1 lacking exon 10 sequences (Bin1-10) (Wechsler-Reya *et al.*, 1997b,1998). Exon 16 was derived from a mouse ES cell genomic fragment generated by PCR, using primers in exon 15 and 16, because none of BAC clones included this exon. Exon 16 sequences shown in Fig. 2 include sequences from the ES subclone as well as from SH3P9 (Sparks *et al.*, 1996).

DNA sequence analysis. The sequence data were assembled manually with assistance from MacVector and AssembLIGN software. Exons were defined by alignment and comparison to the human *BIN1* gene (Wechsler-Reya *et al.*, 1997b) with additional alignments to Bin1 expressed sequence tags in GenBank. Similarity between the mouse and the human coding regions was computed using the BLASTN and TBLASTN algorithms. Promoter sequences were aligned using CLUSTAL W (1.7) and analyzed with a muscle module detection algorithm (Wasserman and Fickett, 1998) that identifies clustered transcription factor binding sites characteristic of muscle-specific promoters.

Immunohistochemistry. Mouse embryos (10.5 days) were fixed for 24 h in 10% neutral buffered formalin, dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks. Five micrometer-thick sections were cut and mounted on Snowcoat X-tra Micro slides (Surgipath), air-dried, and heat-fixed for 30 min at 56°C. Slides were deparaffinized in xylene twice for 10 min each and then rehydrated in decreasing percentages of ethanol, starting at 100% and ending in PBS. Endogenous peroxidase was quenched by incubating for 15 min in 1% H_2O_2 in methanol followed by PBS washing. Slides were then placed in a 600-ml beaker in a slide rack containing 500 ml of 10 mM citrate buffer (pH 8.5), covered with plastic wrap, and microwaved for 5-min intervals for a total of 10 min at the highest power setting (Catoretti *et al.*, 1992). After slides were cooled in the citrate buffer for 20 min, antibody staining was performed essentially as described (Sakamuro *et al.*, 1996). Briefly, tissue was blocked with 10% goat serum and incubated for 30 min with a 1:1500 dilution of Bin1 99I monoclonal antibody from ascites (Wechsler-Reya *et al.*, 1997a). The primary antibody was visualized by a 30-min incubation with biotin-conjugated goat anti-mouse antibody, a 30-min incubation with peroxidase-conjugated streptavidin, and a brief treatment with diaminobenzidine. Before mounting, slides were counterstained with an acidified solution of the cytoplasmic dye light green. Stained embryos were photographed using Kodak T64 film on a Leitz microscope at 80× or 400× magnification.

Western analysis. C2C12 cells expressing antisense Bin1 or containing vector only were cultured in growth or differentiation medium and cell extracts were prepared and processed for Western analysis as described (Wechsler-Reya *et al.*, 1998). Blots were probed with rabbit anti-p21 polyclonal antibody C21 (Santa Cruz Biotechnology) or with murine anti-myosin monoclonal antibody MF20 (obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA). HRP-coupled goat anti-mouse or anti-rabbit IgG (BMB) was used with a commercial chemoluminscence kit (Pierce) to develop the blots. Equal protein loading per lane was subsequently confirmed on blots by staining with Ponceau S.

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). Haplotype analysis of 164 N_2 mice was performed to map the *Bin1* locus; for gene order determination up to 193 mice were typed for some pairs of markers. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins *et al.*, 1982). All blots were prepared with Hybond-N^+ nylon membrane (Amersham). The probe, a 1.4-kb *Eco*RI/*Hin*dIII fragment of mouse *Bin1* cDNA, was labeled with [α-^{32}P]dCTP using a random priming kit (Amersham); washing was performed to a final stringency of 1.0 × SSCP, 0.1% SDS at 65°C. The probe detected 13.0-, 10.5-, 8.0-, 5.0-, 4.6-, and 0.5-kb *Bam*HI fragments in C57BL/6J DNA and 10.0-, 8.0-, 6.6-, 5.4-, 4.7-, 2.7-, and 0.9-kb fragments in *Bam*HI-digested *M. spretus* DNA. The presence or absence of the 6.6-, 5.4-, 2.7-, and 0.9-kb *M. spretus*-specific fragments, which cosegregated, was followed in backcross mice. A description of probes and restriction fragment length polymorphisms (RFLPs) for loci linked to *Bin1*, including desmoglein-3 (*Dsg3*), meprin1, β subunit (*Mep1b*,) and the adenomatosis polyposis coli gene (*Apc*) has been reported previously (Gorbea *et al.*, 1993; Ishikawa *et al.*, 1994). Recombination distances and gene orders were determined using Map Manager, version 2.6.5 (Manly, 1993); gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS AND DISCUSSION

Structure of the Murine Bin1 Gene

A physical map of *Bin1* was determined from a single BAC genomic clone that included the entire locus within an ~100-kb insert (see Fig. 1). The relative location of each exon was determined by Southern analysis, and exon and proximal intron sequences were determined by DNA sequencing (see Fig. 2). The mouse

F1 F2

No.	EXON	INTRON
1	CTCACTCGCTCTCCCCGCGCACGCTCCGTCTCCGTCAGTCCCCTGAGCTG TTCTAGTGCGCGGCGTGGAGCCAGGGCTCAGGCTGGTGGAGCGGCCGGGG CTGGAGGCTGGGAGTGCGGCGCGCACGGCCTCCCCGCGCCATTATCCGCG CTCGCTTCGGGCGAGGCCGGCGCCAGGATGGCAGAGATGGGGAGCAAGGG GGTGACGGCGGGGAAGATCGCCAGCAACGTACAGAAGAAGCTGACCCGAG CGCAGGAGAAG	gtgagtgaacggtaaacctgccacacctctcgcccctaccccgggatct ---- > 10 kb ---- ccggcccctggcttctcaaggtgatgttgcccnttntctctgtgcggcag
2	GTCCTGCAGAAACTGGGGAAGGCGGACGAGACGAAGGACGAGCAGTTTGA GCAGTGTGTCCAGAACTTCAATAAGCAGCTG	gtgagtgtttatggaggtgggacagcgtttgctaggtaggagatggtgag ---- ~ 6 kb ---- ttggtgacagggtccccaggacctgaccttgttcttggctttctggcag
3	ACAGAGGGTACCCGGCTGCAGAAGGATCTTCGGACCTATCTGGCTTCTGT TAAAG	gtagggtacgtctctctgtaaggatttggggctgtcaagctgaggtgggc ---- ~ 150 bp ---- tgtatgagcccgagtggcctggcagggatgaagcaaacaagattgcagag
4	CGATGCACGAAGCCTCCAAGAAGCTGAGTGAGTGTCTTCAGGAGGTGTAT GAGCCCGAGTGGCCTGGCAGGGATGAAGCAAACAAGATTGCAGAG	gtaagcatgggtgggtgcccttggttcttcccccaaggccctttttggctt ---- ~ 300 bp ---- tgcagacagtgtggcttagctctacaaacgctcctgtttctatgtttcag
5	AACAATGACCTACTCTGGATGGACTACCACCAGAAGCTGGTGGACCAGGC TCTGCTGACCATGGACACCTACCTAGGCCAGTTCCCTGATATCAAG	gtaagaaacctctgggcccattgtcttgcttgggttggagcttgtggaag ---- ~ 1.3 kb ---- gggatcttggcaccaggcccnamgatctctcctcctctctgctccctag
6	TCGCGCATTGCCAAGCGGGGGCGGAAGCTGGTGGACTATGACAGTGCCCG GCACCACTATGAGTCTCTTCAAACCGCCAAAAAGAAGGATGAAGCCAAAA TTGCCAAG	gttcccanctgtgggtggggatggtgctgantccagngccacatanaaca ---- > 10 kb ---- ggtagagttccacacagacgctgacgtaccccactgcctctccatcccag
7	GCAGAAGAGGAGCTCATCAAAGCCCAGAAGGTGTTCGAGGAGATGAACGT GGATCTGCAGGAGGAGCTGCCATCCCTGTGGAACAG	gtaaagtcaggaggggcccaggaacctggcgttcagcctggccctgtgtc ---- ~ 200 bp ---- catcccgtcgcatatggttctcaccatgtcacctcctatctctctggcag
8	CCGTGTAGGTTTCTATGTCAACACGTTCCAGAGCATCGCGGGTCTGGAGG AAAACTTCCATAAAGAGATGAGTAAG	gtaggccaggggactgggctgtgcaaaggatcagtcagaggcagggatg ---- ~ 2 kb ---- tgacgaagatgctcgtccaagcctgcttctcttcactcttcctcctgcag
9	CTCAATCAGAACCTCAATGATGTCCTGGTCAGCCTAGAGAAGCAGCACGG GAGCAACACCTTCACAGTCAAGGCCCAACCCAG	gtaggttagggcagggagggtgaggtcagtggggccctgtggcatgatgg ---- ~ 1.5 kb ---- ttcctagctttcttccaaatgaagcatccacactccaaccatccccacag
10	AAAGAAAAGTAAACTGTTTTCGCGGCTGCGCAGAAAGAAGAACAG	gtaccgccttgagtgcagtgccacgggcctctgggccccgccctgactgc ---- ~ 1.9 kb ---- ctggctctttgttctgttgataccactctcggtctgtccttcttttacag
11	TGACAATGCCCCTGAGAAAGGGAACAAGAGCCCGTCACCTCCTCCAGATG GCTCCCCTGCTGCTACCCCTGAGATCAGAGTGAACCATGAGCCAGAGCCG GCCAGTGGGGCCTCACCCGGGGCTACCATCCCCAAGTCCCCATCTCAG	gtaggcacgactgttatctctatgtcctggttttcttctctttctctttc ---- > 6 kb ---- ggatgcatcctgctctgtatctgatccctgctggcattttatgttgcag
13	CCAGCAGAGGCCTCCGAGGTGGTGGGTGGAGCCCAGGAGCCAGGGGAGAC AGCAGCCAGTGAAGCAACCTCC	gtaagacggcaggggccgggccctgttttttcttccctgttgtctgtctg ---- ~ 300 bp gcttttcctacatggccattggtccagctgactcatcccctatccctcag
14	AGCTCTCTTCCGGCTGTGGTGGTGGAGACCTTCTCCGCAACTGTGAATGG GGCGGTGGAGGGCAGCGCTGGGACTGGACGCTTGGACCTGCCCCCGGGAT TCATGTTCAAG	gtgagcgtaggctagccaactctgtagcctttgtctccggtgccttgggg ---- ~ 200 bp tggttaagatgggggaatagccccctganatgccttcttaatttntacag
15	GTTCAAGCCCAGCATGATTACACGGCCACTGACACTGATGAGCTGCAACT CAAAGCTGGCGATGTGGTGTTGGTGATTCCTTTCCAGAACCCAGAGGAGC AG	gtgaacaagggtgtggggaatcccctggctgctgatgcaatggtgggcat ---- ~ 3 kb---- tggtgagtgctgtgtggtgctcctgtgttagccatgctctgttggccccag
16	GATGAAGGCTGGCTCATGGGTGTGAAGGAGAGCGACTGGAATCAGCACAA GGAACTGGAGAAATGCCGCGGCGTCTTCCCGGAGAATTTTACAGAGCGGG TACAGTGACGGAGGAGCCTTCCGGAGTGTGAAGAACCTTTCCCCCAAAGA TGTGTG	

FIG. 2. Exon–intron structure. Exon and proximal intron sequences for the ubiquitously expressed exons within the Bin1 gene are shown. The figure is read from left to right with complete exon sequences shown on the *left* and introns following on the *right*. Register is 50 nt per line. Sizes of intron gaps are approximate based on estimations from restriction mapping.

gene is similar in both structure and organization to the human gene (Wechsler-Reya *et al.*, 1997b), including conservation of a large intron 1 (>20 kb) and a region of ~35 kb that includes the remaining exons. Exons were numbered by reference to the human gene. The mouse *Bin1* gene is ≥55 kb in length, which is similar to the ≥54-kb size of the human gene (Wechsler-Reya *et al.*, 1997b). A full-length mouse Bin1 message, which has been described [SH3P9; (Sparks *et al.*, 1996)], encodes the ubiquitously expressed Bin1 splice form Bin1-10 that includes exons 1–9, 11, and 13–16 (Wechsler-Reya *et al.*, 1997b,1998). The mouse and human coding sequences are ~89% identical at the nucleotide level (mouse nt 41–1461) and ~95% identical at the amino acid level (comparing the ubiquitously expressed Bin1-10 splice isoforms). Proximal intron sequences for each exon were generally highly conserved as well. The BAR (Bin1/Amphiphysin/Rvs167-related) region (Sakamuro *et al.*, 1996) encoded by exons 1–8 includes sequences that are conserved in amphiphysin and conserved in organization to the human Bin1 gene. Exons 9–11 encode the unique region of Bin1 that is not conserved in amphiphysin or RVS167. Exon 9 encodes the unique-1 (U1) region that includes sequences crucial for Bin1 to suppress malignant cell transformation by adenovirus E1A or mutant p53 (Elliott *et al.*, submitted for publication). Exon 10 encodes the unique-3 region (U3) of Bin1 which is alternately spliced following differentiation of C2C12 mouse myoblasts *in vitro* (Wechsler-Reya *et al.*, 1998). U3 was initially thought to encode a nuclear localization signal (Sakamuro *et al.*, 1996) but later investigations argued against this likelihood (Wechsler-Reya *et al.*, 1998). Exon 11 encodes the proline-rich unique-2 (U2) region, which can serve as a pseudosubstrate for the Bin1 SH3 domain (D. Sakamuro, unpublished observations). Murine exons corresponding to brain-specific exons 12A–12D in the human gene (Wechsler-Reya *et al.*, 1997b) were not analyzed in this study. Exons 13 and 14 encode sequences homologous to the Myc-binding domain in human Bin1 (Sakamuro *et al.*, 1996). Exon 13 is alternately spliced in an unregulated fashion in adult and embryonic mouse tissues (Wechsler-Reya *et al.*, 1998; Wechsler-Reya *et al.*, 1997b,).

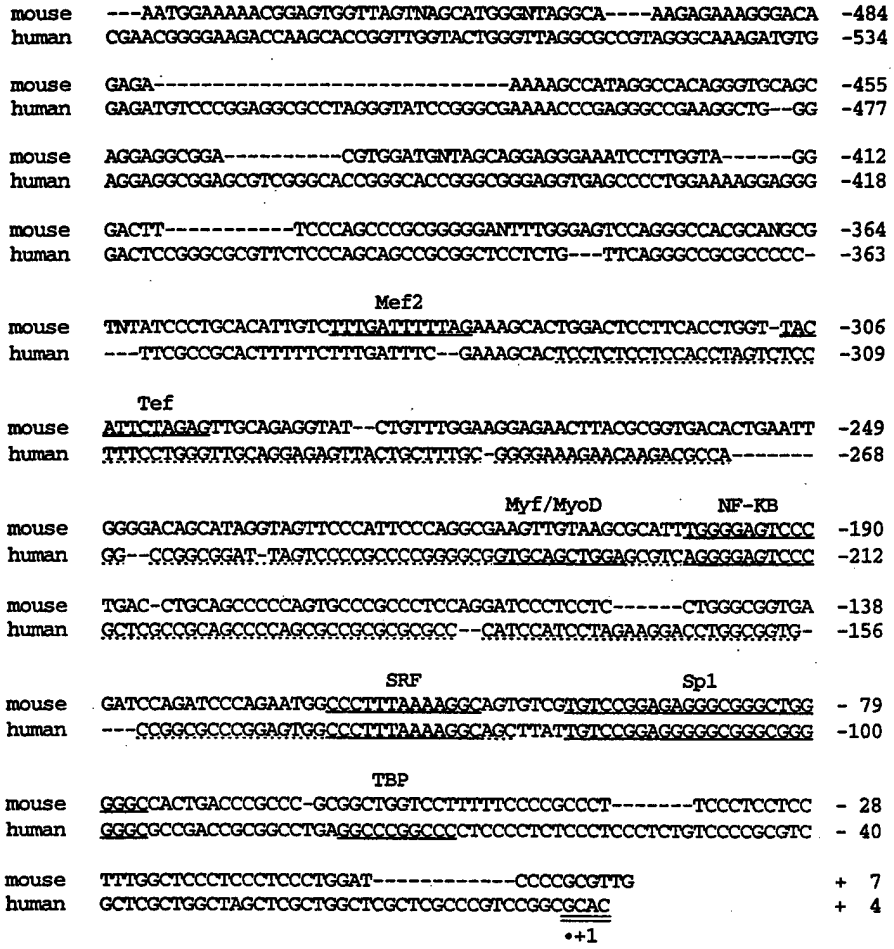

FIG. 3. Structure and conserved regulatory elements of the mouse Bin1 promoter. The DNA sequence of ~0.5 kb of the 5′ flanking region of the mouse gene is shown and aligned with the human promoter. Sequence alignment was performed using CLUSTAL W (1.7) except for the sequences immediately surrounding the human gene cap site (dot) and 5′ end of the mRNA (double underlining), which was aligned by visual inspection. Mouse sequences were numbered by comparison to the human promoter (Wechsler-Reya *et al.*, 1997b). A muscle regulatory module identified by a sequence analysis algorithm (Wasserman and Fickett, 1998) in the human promoter between −330 and −125 is indicated by dotted underlining. Consensus DNA binding sites for various transcription factors identified through this analysis or through visual inspection are noted by solid underlining. Mef2 and Tef consensus sites in the mouse were not conserved in the human promoter, whereas a strong Myf/MyoD consensus site in the human was not conserved in the mouse. A site analogous to the TBP binding site noted in the human promoter (Wechsler-Reya *et al.*, 1997b) was also not detected in the mouse sequence.

Exons 15 and 16 encode the Src homology 3 domain of Bin1, a feature that is shared with amphiphysin and RVS167 (Sakamuro *et al.*, 1996) and that is necessary for interaction with c-Abl and dynamin (Kadlec and Pendergast, 1997; Owen *et al.*, 1998).

Conserved Features of the Bin1 Promoter Suggest Roles in Myogenesis and Apoptosis

The DNA sequence of the 5′ flanking region upstream of exon 1 was determined, and this region was analyzed and compared with the human promoter (see Fig. 3). There was significant conservation between the mouse and the human 5′ flanking regions within 400 bp of exon 1, consistent with the identification of the mouse *Bin1* promoter. Bin1 is expressed robustly in skeletal muscle from adult mouse and human (Sakamuro *et al.*, 1996), so the 5′ flanking regions of each gene were analyzed using algorithms that identify transcription factor consensus binding sites and muscle regulatory modules (Wasserman and Fickett, 1998). A muscle regulatory module was identified in the human promoter between −330 and −125 (Fig. 3, dotted underline) but an analogous module was not detected in the mouse promoter. This absence suggested that there may be some difference in how the human and mouse genes are regulated during differentiation. Consistent with the latter possibility, the mouse region also lacked a CpG island that is present in the human promoter (Wechsler-Reya *et al.*, 1997b). The muscle regulatory sites identified in the human module included myogenin/myoD, Sp1, and serum response factor (SRF). The role of the myoD family b/HLH transcription factors in directing muscle differentiation is well known. SRF was initially identified as

an activator of the c-*fos* promoter but was later found to be crucial for regulating skeletal muscle-specific and smooth muscle-specific gene expression (Duprey and Lesens, 1994). In the 5′ flanking region of the mouse gene, the Sp1 and SRF sites were conserved but not the myogenin/myoD site, pointing to another difference with the human promoter. In place of the myogenin/myoD site were weak sites for Mef2 and Tef. Mef2 is a MADS-box transcription factor that is required for muscle development (Olson *et al.,* 1995). Since *Bin1* must be upregulated for differentiation of C2C12 myoblasts (Wechsler-Reya *et al.,* 1998), the Mef2 site may be relevant to this event because it has also been shown to be required for C2C12 differentiation (Ornatsky *et al.,* 1997). The Mef site may also be relevant to the high-level expression of *Bin1* in adult brain (Butler *et al.,* 1997; Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1997b) and in PC12 cells (R. Wechsler-Reya and G.C.P., unpublished results) since Mef2 isoforms are strongly expressed in neuronal cells (Lyons *et al.,* 1995). The Tef site may be relevant to housekeeping as well as muscle-specific regulation of Bin1. Tef was originally identified as an SV40 enhancer-binding factor but was subsequently discovered to be an important factor in cardiac muscle-specific gene regulation (Farrance *et al.,* 1992). Sp1 and Tef sites are proximal in the SV40 promoter so interactions between these factors may be involved in the basal but ubiquitous *Bin1* expression seen in tissues outside of muscle and brain (Wechsler-Reya *et al.,* 1997b). Taken together, the data suggested that SRF and Sp1 directed muscle-specific expression of *Bin1* with additional contributions from myogenin/myoD in human and from Tef and Mef2 in mouse.

One notable feature of the *Bin1* promoter identified in this study was the presence of an evolutionarily conserved strong consensus binding site for NF-κB. This finding was interesting because of evidence that Bin1 has a positive role in c-Myc-mediated apoptosis and that Bin1 can drive apoptosis of tumor cells that contain deregulated c-Myc (D. Sakamuro, K. Elliott, K. Ge, J. Duhadaway, D. Ewert, and G.C.P., manuscripts in preparation). NF-κB has important roles in oncogene-mediated cell transformation (Mayo *et al.,* 1997; Reuther *et al.,* 1998) and apoptosis (Baichwal and Baeuerle 1997). The likelihood that the NF-κB site in the Bin1 promoter is functional was supported by the observations that (1) Bin1 message levels were increased by TNF-α, which stimulates NF-κB activity, and that (2) a Bin1 promoter-reporter gene could be activated several-fold by TNF-α or RelA/p50 and c-Rel/p50 in transient cotransfection assays (data not shown). In future work, it will be important to determine whether Bin1 mediates certain NF-κB responses in apoptosis, for example, those activated by tumor necrosis factor, and whether the Myc-Bin1 system may modulate the ability of NF-κB to control apoptosis.

Bin1 Is Upregulated during Development by E10.5 in Myotomes

The inability to identify a muscle regulatory module in the mouse *Bin1* promoter prompted us to investigate the expression of *Bin1* during muscle development. Myogenesis initiates during development in somites, segmented paraxial mesoderm that is arrayed along the dorsal axis alongside the developing central nervous system. The dorsal part of the somite includes the myotome, which is the progenitor of skeletal muscle. Early stages of myogenesis are apparent in E10.5 myotomes because myf5 and myoD have been switched on and elongation of cells destined to become muscle can be seen. We performed an immunohistochemical analysis of E10.5 embryos with a Bin1 monoclonal antibody (Wechsler-Reya *et al.,* 1997a) to determine whether *Bin1* was switched on at this stage. In sagittal sections, strong staining was detected in elongated cells present in a dorsally located segmented pattern consistent with somites (see Figs. 4A and B). The cytoplasmic staining pattern was consistent with that seen following *in vitro* differentiation of C2C12 myoblasts, when Bin1 is exported from the nucleus to the cytosol (Wechsler-Reya *et al.,* 1998). In transverse sections, staining was confined to a medial part of a dorsolateral segment of the myotome (see Fig. 4C, arrowhead, and Fig. 4D). Staining was specific in the somite region inosfar as there was no significant staining of the adjacent sclerotomes, which are the progenitors of skeletal bone. The data indicated that *Bin1* was activated during myogenesis even though its promoter lacked a consensus DNA binding site for the important myoD family of muscle determination factors (Molkentin and Olson, 1996). Mef2 is expressed before E10.5 in the myotome (Edmondson *et al.,* 1994) so this factor may be responsible for activating the *Bin1* gene at this time. We concluded that despite the absence of a myoD family consensus binding site in its promoter, *Bin1* was activated at an early stage of myogenesis.

F4

Bin1 Functions at an Early Time during Myoblast Differentation

In previous work, we showed that Bin1 is induced and has a necessary role during differentiation of C2C12 myoblasts (Wechsler-Reya *et al.,* 1998), a model for muscle differentiation *in vitro.* The immunochemical results above validated the induction of Bin1 seen in C2C12 and raised the question of when Bin1 acts during myoblast differentation. We have shown that C2C12 cells expressing antisense Bin1 do not exit the cell cycle and differentiate following serum deprival (Wechsler-Reya *et al.,* 1998). An important early event in C2C12 differentiation that leads to cell cycle inhibition is induction of the cell cycle kinase inhibitor $p21^{WAF1}$ (Walsh and Perlman, 1997). Therefore, we examined the regulation of $p21^{WAF1}$ or myosin, a marker for biochemical differentiation, in antisense or control C2C12 cell lines generated previously.

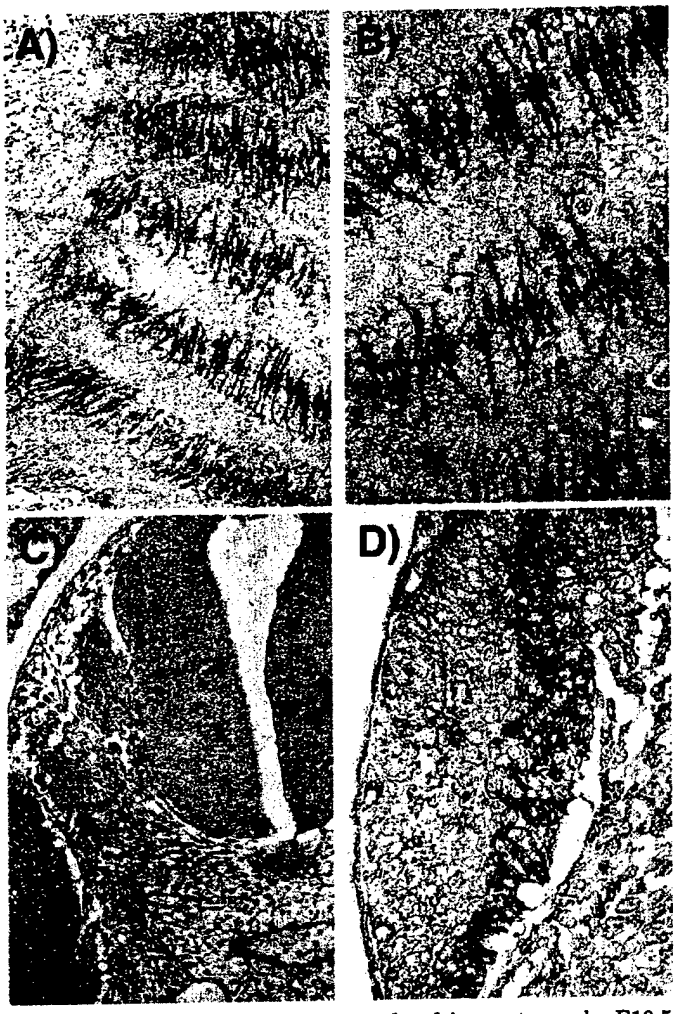

FIG. 4. *Bin1* is specifically upregulated in myotomes by E10.5 during murine development. E10.5 embryos were processed for Bin1 immunohistochemistry as described under Materials and Methods. (**A**) Sagittal section illustrating expression of *Bin1* in elongating myoblasts in somites, 80×. (**B**) Same section as above, 400×. (**C**) Transverse section illustrating expression of *Bin1* in the myotome but not the sclerotome of a somite (arrowhead), 80×. Orientation is provided by the neural tube seen in the upper right corner of the figure. Expression of *Bin1* in endothelial cells (bottom right side of the figure) and in certain neurons in the neural tube, a phenomenon also noted in the brain (data not shown), is also illustrated in this figure. (**D**) Same section as above, 400×.

Western analysis of extracts isolated at various times after induction of differentiation by serum deprival showed that $p21^{WAF1}$ was not appropriately upregulated in antisense cells (see Fig. 5). In control cells, $p21^{WAF1}$ levels steadily increased from a basal level of expression starting at day 1 after serum deprival. Myosin expression indicative of complete biochemical differentiation was first detected at day 3 in these cells. In contrast, in cells expressing antisense Bin1, $p21^{WAF1}$ was undetectable in undifferentated cells (day 0) and was only transiently induced on day 1 after differentiation was induced. As seen before, these cells did not proceed to express myosin and biochemically differentiate (Wechsler-Reya *et al.*, 1998), presumably because $p21^{WAF1}$ was not appropriately upregulated such that cells could exit the cell cycle. We concluded that Bin1 functioned at an early stage of myoblast differentiation, at a point required to sustain activation of $p21^{WAF1}$ and subsequent cell cycle exit.

Bin1 Is Located within the Proximal Region of Mouse Chromosome 18

The chromosomal location of *Bin1* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2500 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). To identify informative RFLPs for gene mapping, the mouse *Bin1* cDNA was used as a probe in Southern blot analysis of C57BL/6J and *M. spretus* genomic DNAs digested with several restriction enzymes (see Materials and Methods). The inheritance of the *M. spretus*-specific alleles was followed in backcross mice, and the strain distribution pattern of the RFLP was determined to position the locus on the interspecific backcross map. The mapping results indicated that the *Bin1* locus is located in the proximal region of chromosome 18 (see Fig. 6), 0.5 cM proximal to *Apc*. The same results were obtained by using *Hin*cII polymorphisms and by using the human *BIN1* cDNA as a probe to follow *Taq* I polymorphisms (data not shown). We compared our interspecific map of chromosome 18 with composite mouse linkage maps that report the map location of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME).

In humans, *BIN1* has been mapped to chromosome 2q14 by fluorescence *in situ* hybridization and by PCR analysis of somatic cell hybrids (Negorev *et al.*, 1996), but synteny has not been reported previously between human chromosome 2q14 and mouse chromosome 18. However, in the mouse, synteny has not been deter-

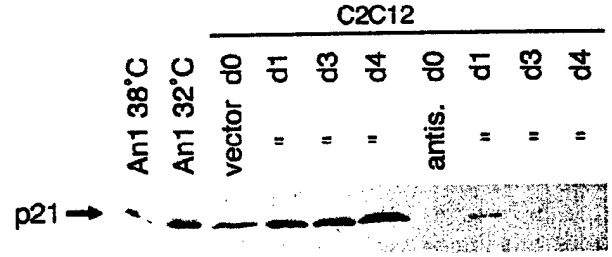

FIG. 5. Impaired activation of $p21^{WAF1}$ in myoblasts whose differentiation is blocked by antisense Bin1. Cell extracts were prepared from control or antisense Bin1-expressing C2C12 murine myoblasts (Wechsler-Reya *et al.*, 1998). Cells were cultured in growth medium (day 0, d0) or in differentiation medium for various times (d1,d3, and d4) before extract preparation. Western blotting was performed using anti-p21 and anti-myosin antibodies. A control for p21 induction was provided by the rat cell line BRK/An1, which harbors a temperature-sensitive p53 mutant; in this cell lines p21 is induced by activation of wildtype p53 at 32°C but not mutant at 38°C.

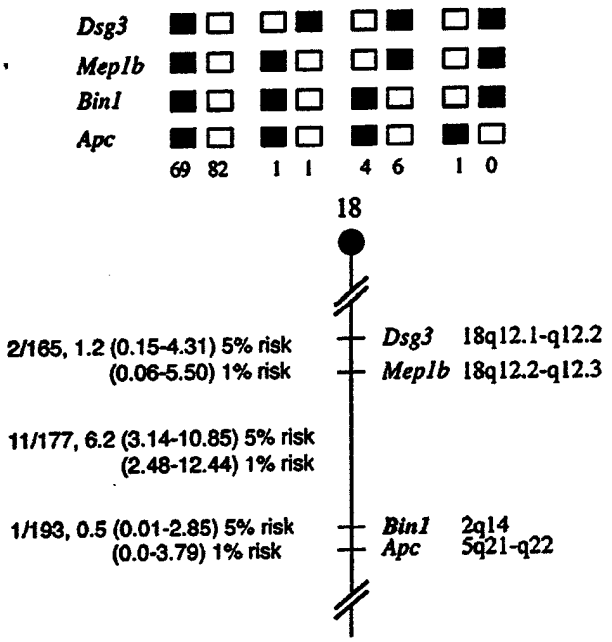

FIG. 6. Partial chromosome linkage map showing the mouse chromosomal location of *Bin1* as determined by interspecific backcross analysis. (**Top**) Segregation patterns of *Bin1* and flanking genes. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. Black boxes indicate the presence of a C57BL/6J allele, and white boxes indicate the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column, for a total of 164 mice analyzed for the segregation analysis of *Bin1*. (**Bottom**) Gene order analysis. Data from up to 193 mice were used to generate the partial chromosome linkage map of chromosome 18, which indicated the location of *Bin1* in relation to linked genes. To the left of the chromosome map is shown the number of recombinant N_2 animals over the total number of animals typed, with the recombination frequencies for each pair expressed as genetic distances in centimorgans (with confidence intervals at the 5 or 1% risk level). The positions of loci in human chromosomes are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

mined in the 6.7-cM region between loci that flank *Bin1* and have been mapped in both species (*Mep1b* and *Apc*). As discussed above, the human and mouse genes share significant similarity, and all the *Bin1* polymorphisms that we followed in backcross mice fell into the same region on mouse 18. Therefore, we conclude that the *Bin1* locus truly defines a new region of synteny.

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BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression

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We have identified a novel protein, BAP1, which binds to the RING finger domain of the Breast/Ovarian Cancer Susceptibility Gene product, BRCA1. BAP1 is a nuclear-localized, ubiquitin carboxy-terminal hydrolase, suggesting that deubiquitinating enzymes may play a role in BRCA1 function. BAP1 binds to the wild-type BRCA1-RING finger, but not to germline mutants of the BRCA1-RING finger found in breast cancer kindreds. *BAP1* and *BRCA1* are temporally and spatially co-expressed during murine breast development and remodeling, and show overlapping patterns of subnuclear distribution. *BAP1* resides on human chromosome 3p21.3; intragenic homozygous rearrangements and deletions of BAP1 have been found in lung carcinoma cell lines. BAP1 enhances BRCA1-mediated inhibition of breast cancer cell growth and is the first nuclear-localized ubiquitin carboxy-terminal hydrolase to be identified. *BAP1* may be a new tumor suppressor gene which functions in the BRCA1 growth control pathway.

Keywords: ubiquitin hydrolase; BRCA1; chromosome 3p21.3; RING finger

Introduction

The cloning of the chromosome 17q21 *BRCA1* breast cancer susceptibility gene is a landmark accomplishment in cancer genetics (Miki *et al.*, 1994). Germline mutations in *BRCA1* appear to account for ~50% of familial breast cancers and essentially all families with 17q21-linked inherited susceptibility to ovarian and breast cancer (Szabo and King, 1995). The importance of this gene is underscored by the fact that kindreds segregating constitutional *BRCA1* mutations show a lifetime risk of 40–50% for ovarian cancer and >80% for breast cancer (Easton *et al.*, 1993, 1995). The classification of *BRCA1* as a highly penetrant, autosomal dominant tumor suppressor gene has been genetically confirmed by the finding of frequent LOH of the wild-type allele in breast tumors from mutation carriers (Hall *et al.*, 1990; Miki *et al.*, 1994; Smith *et al.*, 1992). Surprisingly, *BRCA1* mutations in sporadic breast cancer, including those which show 17 g LOH, have yet to be found and *BRCA1* mutations are extremely rare in sporadic ovarian cancer (Futreal *et al.*, 1994; Merajver *et al.*, 1995).

The *BRCA1* locus spans >100 kb comprising 24 exons (Miki *et al.*, 1994). More than 100 constitutional mutations have been identified in *BRCA1* over the entire length of the gene. Some clustering of these mutations has been seen in populations, and genotype-phenotype correlations have been suggested (FitzGerald *et al.*, 1996; Ford *et al.*, 1994; Muto *et al.*, 1996; Roa *et al.*, 1996; Struewing *et al.*, 1995). The majority of germline mutations result in a truncated BRCA1 protein although recurrent missense mutations resulting in amino acid substitutions in kindreds have also been observed (Couch and Weber, 1996). The heterogeneity of BRCA1 mutant proteins produced by this spectrum of genetic mutations suggests that multiple, independent functions and/or protein–protein interaction surfaces are targets for mutational inactivation. However, the biochemical functions of BRCA1 are largely unknown.

The predominant *BRCA1* mRNA of 8.0 kb encodes a 1863 amino acid protein with only a few sequence motifs suggestive of function (Miki *et al.*, 1994). There are two highly conserved regions. The first is the 100 amino acid N-terminus which encodes a RING finger motif, a domain that is predicted to bind zinc and may be a protein–protein interaction motif (Borden *et al.*, 1995; Lovering *et al.*, 1993). The second region is at the C-terminus which contains an acidic region and two copies of a novel motif, designated the BRCT domain. The BRCT domain is present in a variety of putative cell-cycle related proteins, including RAD9 and 53BP1 (Koonin *et al.*, 1996). The most abundant BRCA1 protein is apparently a ~220 kDa phosphoprotein which is predominantly, but apparently not exclusively, nuclear in subcellular distribution (Chen *et al.*, 1995, 1996b; Scully *et al.*, 1996). BRCA1 is localized to discrete nuclear dot structures in a cell-cycle-dependent manner (Scully *et al.*, 1997b). Other isoforms of BRCA1 have been detected including a protein of 97 kDa. This smaller form lacks exon 11, and thus a functional nuclear localization signal, and is presumably the result of an alternative splicing event (Thakur

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et al., 1997). The above observations, coupled with the finding of a BRCA1 COOH-terminal domain capable of activating transcription as a Gal4 DNA-binding domain fusion (Chapman and Verma, 1996) and the co-fractionation of BRCA1 with the RNA pol II holoenzyme (Scully *et al.*, 1997a), suggest a role for BRCA1 in transcriptional regulation.

The expression patterns of *BRCA1* further support its role in growth regulation and/or differentiation. The spatial-temporal expression pattern in the embryonic mouse includes the neuroepithelium, and epithelial lineages of the skin, kidney and mammary gland (Marquis *et al.*, 1995). Moreover, *BRCA1* mRNA is sharply increased in alveolar and ductal cells of the breast epithelia during pregnancy (Marquis *et al.*, 1995). Consistent with this, *BRCA1* transcription is under (indirect) hormonal control in both cell culture and organismal systems (Gudas *et al.*, 1995, 1996; Vaughn *et al.*, 1996; Marks *et al.*, 1997). *BRCA1* is also highly expressed in the adult testis during the final stages of meiosis and spermiogenesis (Zabludoff *et al.*, 1996). Together, these observations suggest a broad role for BRCA1 in terminal differentiation events in multiple tissues. Somewhat paradoxically, the murine *brca1*−/− embryos die very early in gestation and exhibit severe cell proliferation defects and profound cell cycle arrest (Hakem *et al.*, 1996; Liu *et al.*, 1996). The association of *BRCA1* expression with both proliferation and differentiation events suggests a possible role for BRCA1 in regulating a genetic program which prepares the cell for terminal differentiation and possibly maintains that phenotype. Results of cell culture and transfection studies have underscored the tumor suppression function of BRCA1, but have revealed little of possible mechanisms. *BRCA1* antisense expression can transform fibroblasts and accelerates growth of breast cancer cell lines (Rao *et al.*, 1996; Thompson *et al.*, 1995). Expression of wild-type *BRCA1* inhibits colony formation and tumor growth *in vivo*, whereas tumor derived mutants of *BRCA1* lack this growth suppression activity (Holt *et al.*, 1996).

Evidence of a role for BRCA1 as a terminal differentiation checkpoint has recently been provided by the finding that BRCA1 and the RAD51 protein (involved in DNA recombination/repair) are co-localized and physically associated in mitotic and meiotic cells (Scully *et al.*, 1997b). The co-localization of BRCA1 and RAD51 on synaptonemal meiotic chromosomes suggests a role for this complex in either the fidelity of DNA replication, cell-cycle progression or genomic integrity. Though intriguing, these results do not suggest a function for BRCA1 which, when lost through mutation of the BRCA1 gene, would give rise to tumors. Strategies based upon identification of proteins which bind to BRCA1 have yielded components of the nuclear import pathway (Chen *et al.*, 1996a) and a novel RING finger/BRCT-domain-containing protein, BARD1 (Jin *et al.*, 1997; Wu *et al.*, 1996). However, none of these associated proteins have suggested a function for BRCA1.

We have chosen to focus upon the highly conserved BRCA1 RING finger domain as a potential protein–protein interface. This motif is defined by a spatially conserved set of cysteine–histidine residues of the form C_3HC_4. Structural analysis of the motif shows that two molecules of zinc are chelated by the consensus residues in a unique 'cross-braced' fashion (for reviews, see; Klug and Schwabe, 1995; Saurin *et al.*, 1996). Comparative structure analyses suggest that the RING fingers have a common hydrophobic core structure but that the region encoded by amino acids spanning cysteines 24 and 64 (for BRCA1) forms a highly variable loop structure which may be the determinant of protein–protein interaction specificity. The RING motif occurs in over 80 proteins including the products of proto-oncogenes and putative transcription factors (Saurin *et al.*, 1996). Evidence that the RING finger domain functions as a protein–protein interface has come from the study of the proto-oncogene PML (Borden *et al.*, 1995) and the transcriptional co-repressor KAP-1 (Friedman *et al.*, 1996). Intriguingly, like BRCA1, both PML and KAP-1 are localized to discrete, non-overlapping, nuclear dot structures, and mutations in the RING finger of PML abolish its localization to these dot structures (Borden *et al.*, 1995).

We hypothesize that the BRCA1 RING finger is a binding site for protein(s) which either mediate BRCA1 tumor suppressor function or serve to regulate these functions. Genetic evidence supports this in that single amino-acid substitutions at metal chelating cysteines, C61G and C64G, occur in *BRCA1* kindreds; these mutations segregate with the disease susceptibility phenotype and are predicted to abolish RING finger structure. We have used the yeast two-hybrid system to isolate proteins which bind to the wild-type BRCA1 RING finger but not to the C61G or C64G mutated RING fingers or other closely related RING fingers. We have isolated mouse and human clones of a novel protein, *B*RCA1 *a*ssociated *p*rotein-1 (BAP), which fulfils these criteria. BAP1 is a novel, nuclear-localized enzyme which displays the signature motifs and activities of a ubiquitin carboxy-terminal hydrolase. Full-length BRCA1 binds to BAP1 *in vitro* and enhances the growth suppression properties of BRCA1 in colony formation assays. The human *BAP1* locus was mapped to chromosome 3p21.3, and homozygous deletions of *BAP1* were found in non-small cell lung cancers. Together, these data suggest that *BAP1* is a key player in the *BRCA1* growth suppression pathway, and may itself be a tumor suppressor gene. The identification of BAP1 as a ubiquitin hydrolase implicates the ubiquitin-proteasome pathway in either the regulation, or as a direct effector, of BRCA1 function. BAP1 is the first nuclear-localized ubiquitin carboxy-terminal hydrolase to be identified, and may play a broad role in ubiquitin-dependent regulatory processes within the nucleus, including the emerging roles of ubiquitin conjugation as a post-translational modification which alters protein function and/or subcellular targeting.

Results

A yeast two-hybrid screen for BRCA1 RING finger interacting proteins

We constructed a synthetic *BRCA1* gene encoding the amino-terminal 100 amino acids of human BRCA1 using long oligonucleotides and PCR-mediated over-

lap-extension gene synthesis techniques (Madden *et al.*, 1991). Codon usage was optimized for expression in *E. coli* and *S. cerevisiae*. The resulting gene was fused to the LexA DNA binding domain (Figure 1b). The negative control/specificity controls included: (1) the Cys61Gly, Cys64Gly, Met18Thr and Arg71Gly muta-

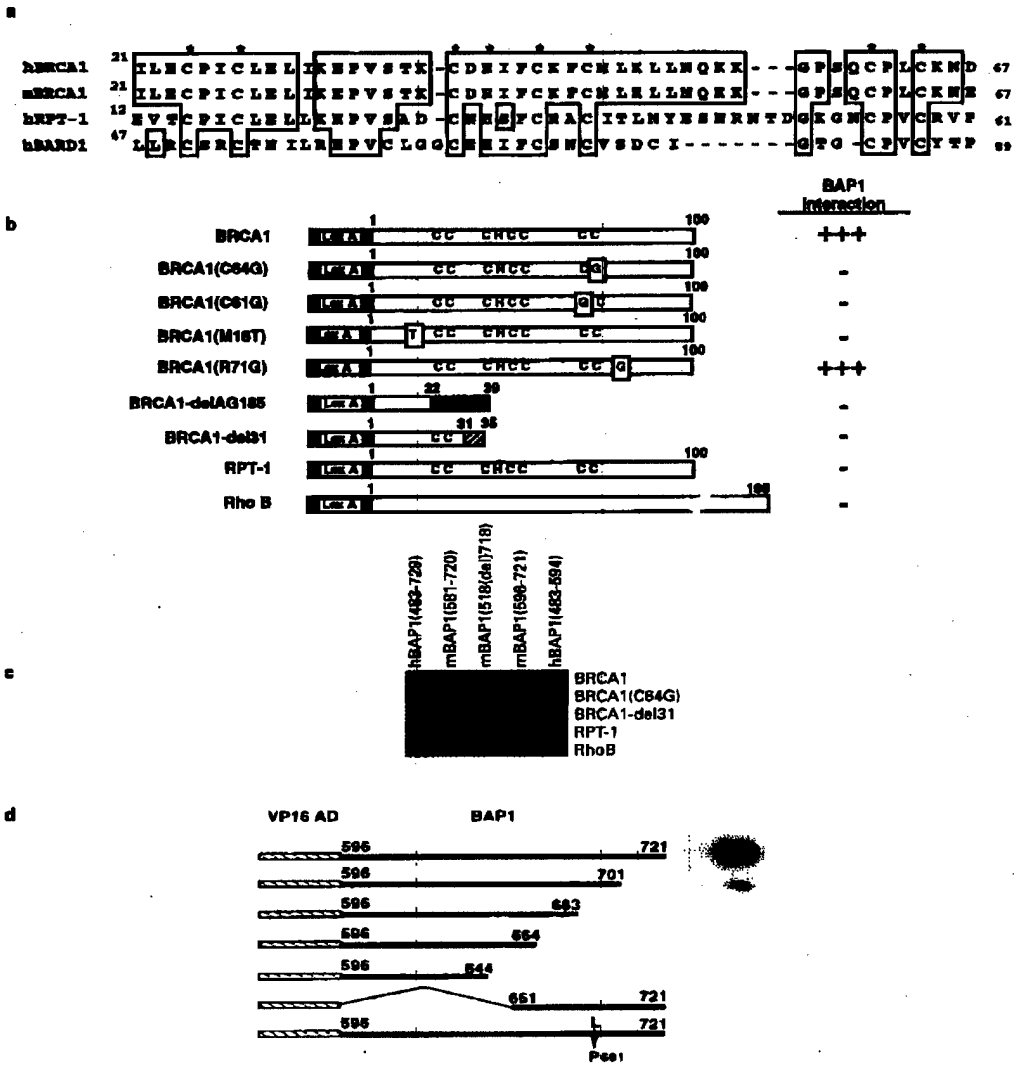

Figure 1 BRCA1 RING finger domain binds a novel protein. (**a**) Alignment of the RING finger domains of human and mouse BRCA1 (AA's 21–67), RPT-1 (AA's 12–61; the most closely related RING finger) and BARD1 (AA's 47–89). Asterisks (*) identify the Zn-chelating amino acids that form the core of the RING finger. Boxed amino acids show regions of identity between the RING finger domains of human BRCA1 and the other proteins. Alignment performed by CLUSTALW (Thompson *et al.*, 1994). (**b**) The amino-terminal 100 amino acids of human BRCA1 (which includes the RING finger domain) or the indicated amino acids of the various BRCA1–RF mutants and controls were fused to the LexA DNA-binding domain. Expression of all fusions in yeast was confirmed by Western analysis. A summary of the two-hybrid interaction between the Gal4-hBAP1(483–729) fusion clone and the various LexA-RING finger fusions is shown. (**c**) The BRCA1-interacting protein specifically interacts with the BRCA1 RING finger domain. Two hybrid screens of a human B-cell library and a mouse embryo (9.5–10.5 days) library identified a protein that interacted with wild type BRCA1–RF, but not with BRCA1-del31 (a truncated BRCA1), BRCA1(Cys64Gly) (a BRCA1–RF containing a point mutation), RPT-1 (a RING finger closely resembling the BRCA1), or RhoB (a non-related protein). Dark color of yeast indicates transcription from the LacZ reporter gene. Clones obtained from the two libraries are described as partial BAP1 proteins with AAs in parentheses. h, human; m, mouse. (**d**) The two-hybrid interaction between the BRCA1 RING domain and BAP1 requires the BAP1 C-terminal domain. Murine clone mBAP1(596–721) defines a portion of the BRCA1-interaction domain of BAP1. Mutants of this clone were generated by PCR-based deletion or point mutagenesis of mBAP1(596–721) as described in Materials and methods. Each individual mutant was co-transformed with LexA-BRCA1–RF into yeast and tested for interaction via its ability to activate transcription from the LacZ locus. Expression of all fusions in yeast were confirmed by Western analysis

tions that occur in *BRCA1* families; (2) the protein equivalent of the del AG185 mutation which results in a frame-shift at amino acid 22 followed by 17 out-of-frame amino acids and a stop codon; (3) a truncated BRCA1 RING finger at amino acid 31, the result of a PCR error; (4) the RPT-1 RING finger domain, a putative lymphocyte-specific transcription factor, whose RING finger domain is most highly related to

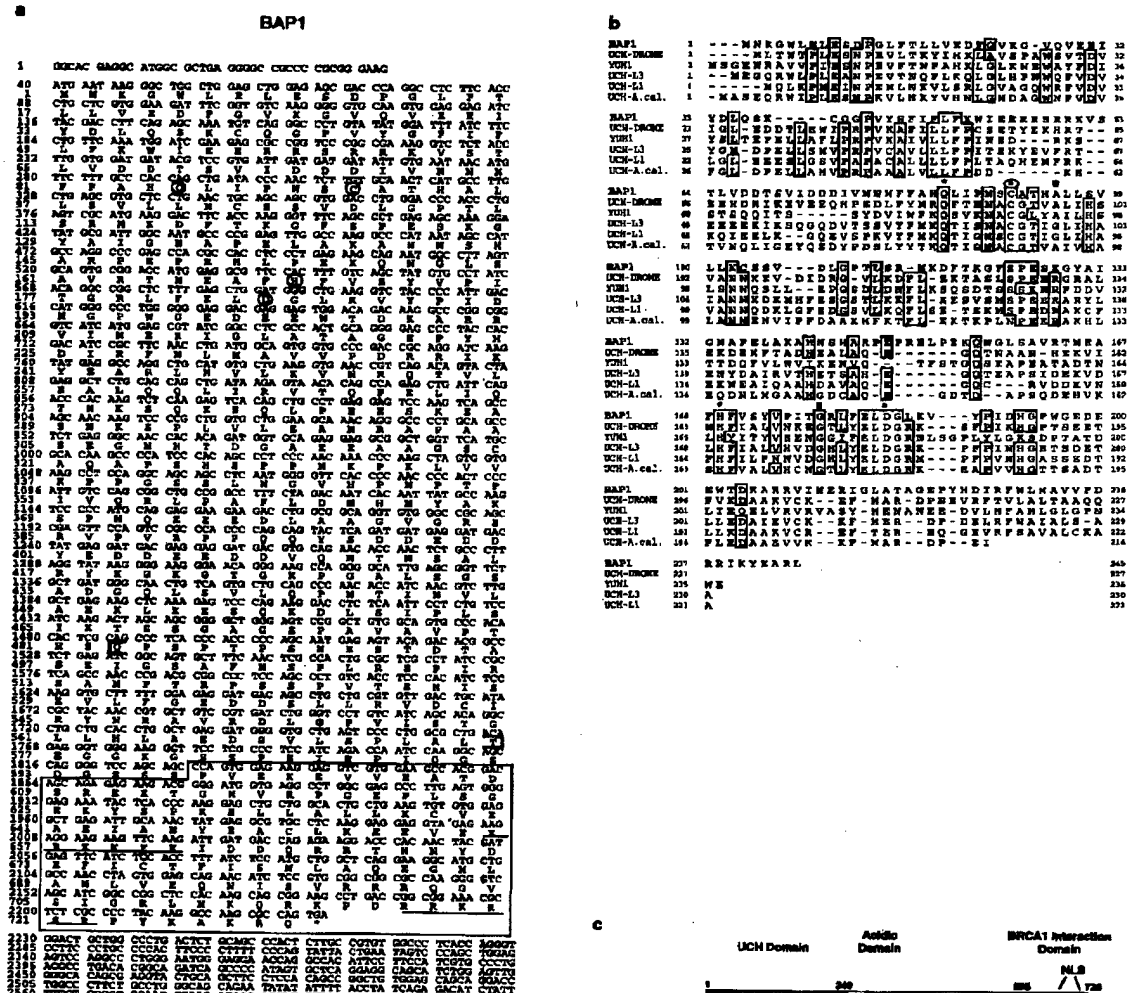

Figure 2 BAP1 is a novel ubiquitin carboxy-terminal hydrolase (UCH). (**a**) The nucleotide and amino acid sequence of BAP1. The longest open reading frame which contained the amino acids defined by the human 2-hybrid fusion protein is 2188 nucleotides encoding 729 amino acids. The cDNA also contains 39 nucleotides of 5'UTR and 1295 nucleotides of 3'UTR. The enzymatic active site is contained within the first 250 amino acids; the active site residues are circled. The putative nuclear localization signals (NLS) are underlined, the BRCA1-interaction domain is boxed and the protein fragment used to generate BAP1 polyclonal antibodies is bracketed (AAs 483–576). (**b**) Comparison of BAP1 with other UCH's. UCH DROME (genebank #P35122), YUH1 (genebank #P35127), UCHL-1 (genebank #PO9936), UCHL-3 (genebank #P15374), UCH A.cal. (genebank #U90177). The BLAST search algorithm was used to identify proteins closely related to BAP1 (Altschul *et al.*, 1990). The UCH domain of five of these proteins were aligned with BAP1 using the CLUSTALW (ver.1.6) algorithm (Thompson *et al.*, 1994). Residues conserved between species with an identity of 85% or greater are boxed. Active site residues are denoted with asterisks. The catalytic cysteine is marked with a circled asterisk. (**c**) Diagrammatic comparison between BAP1 and some of the other UCH family members

that of BRCA1 (Patarca *et al.*, 1988); and (5) a LexA fusion with RhoB. The wild-type BRCA1 RING finger (BRCA1–RF) did not display intrinsic transcriptional activation function in yeast, and expression of each LexA fusion in yeast was confirmed by Western blot analysis with anti-LexA antibody (data not shown).

Guided by the expression patterns of BRCA1 during mouse development and in human spleen, we chose to screen cDNA libraries constructed from E9.5-10.5d whole mouse embryos and human adult B cells with the LexA-BRCA1–RF. Thirty-one cDNAs which specifically interacted with BRCA–RF were obtained: eight of these (three from the human library and five from the mouse library) encoded the same amino acid sequence. These were designated *BRCA1 Associated Protein-1* (BAP1) and pursued further. Each clone shares the same translational reading frame with respect to the transcriptional activation domain to which it is fused. In addition, the fusion junctions were different among the clones, suggesting that the interaction was not due to a fusion-junction artifact. Furthermore, the hBAP1 (483–729) and the BRCA1–RF interacted strongly in a mammalian two-hybrid assay (data not shown). The longest *BAP1* cDNA retrieved in the two-hybrid screen was a ~2.0 kbp clone from the human library and encoded 246 amino acids followed by a 1.3 kb 3'UTR. Each murine clone encoded an overlapping, smaller subset of this human open reading frame with a human-murine AA sequence identity of 100% over the COOH-terminal 100 AAs (data not shown). Both human and mouse clones showed a strong interaction with the wild-type BRCA1–RF and BRCA1 (Arg71Gly) substitution, but failed to interact with the C64G, C61G, del31, delAG, RPT-1, RhoB, or a variety of other LexA fusion constructs (Figure 1b,c and data not shown).

Further definition of this highly conserved interaction domain was performed by mutagenesis of this region of BAP1. Deletion of protein sequence from the carboxyl or amino termini of mBAP1(596–721) almost completely destroyed the BAP1-BRCA1 interaction (Figure 1d), possibly suggesting an extended interface between the proteins. Interestingly, the mBAP1(518del718) clone interacted most poorly with BRCA1–RF (Figure 1c) and lacked a 93 bp sequence (the reading frame was maintained), possibly the result of a naturally occurring splice variant. That BAP1 also failed to bind multiple, independent tumor-derived mutations of the BRCA1–RF provides strong evidence for the relevance of this interaction to the functions of BRCA1.

Analysis of the BAP1 cDNA

A full-length cDNA was constructed using two IMAGE consortium EST clones and RT–PCR (Figure 2a; see Materials and methods). The *BAP1* cDNA comprises 3525 bp; a polyA tract is present along with multiple polyA signals. Conceptual translation yields a long open reading frame of 729 amino acids with a predicted MW of 81 kDa and pI of 6.3. The presumptive initiator methionine is within a favorable context for translation start, however the short 5'UTR of 39 bp encodes amino acids in-frame with the presumptive methionine and does not contain

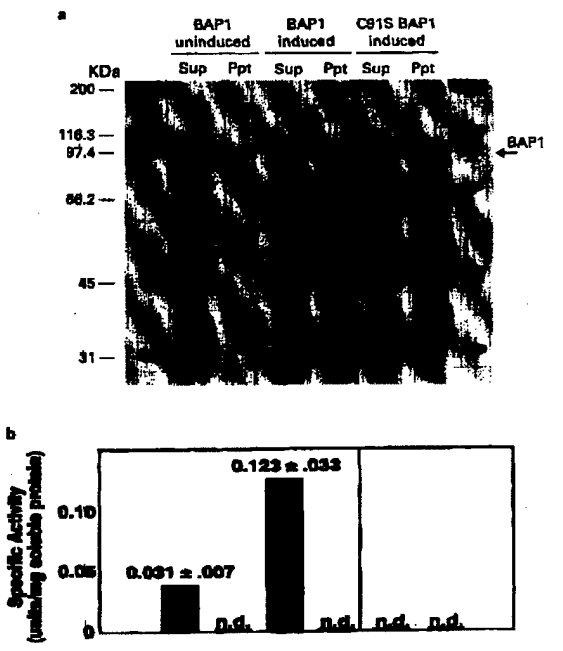

Figure 3 BAP1 has ubiquitin hydrolase activity. BAP1, or an enzymatically null mutant, BAP1(C91S), were expressed in bacteria by IPTG induction. Bacteria were harvested, lysed and supernatant (Sup) and pellet (Ppt) fractions generated. Each fraction was then measured for UCH activity (bar diagram; n.d., not detected). Induction of protein was verified by SDS–PAGE of each fraction. Arrow indicates BAP1 and BAP1(C91S) protein

a stop codon. Computer database searches indicated that BAP1 is a novel protein with the amino-terminal 240 amino acids showing significant homology to a class of thiol proteases, designated ubiquitin C-terminal hydrolases (UCH), which are implicated in the proteolytic processing of ubiquitin (Wilkinson *et al.*, 1989). These enzymes play a key role in protein degradation via the ubiquitin-dependent proteasome pathway. Similarities to other mammalian UCHs (UCH-L3 and UCH-L1) have been found (Figure 2b and c). Most importantly, the residues which form the catalytic site (Q85, C91, H169 and D184) are completely conserved, including the FELDG motif (Larsen *et al.*, 1996). In addition, a loop of highly variable sequence, which is disordered in the crystallographic structure of human UCH-L3 (Johnston *et al.*, 1997), is present (residues 140–167). This loop may occlude the active site or provide substrates specificity for the enzyme.

BAP1 has a number of additional motifs; a region of extreme acidity spanning amino acids 396 to 408, as well as multiple potential phosphorylation sites and N-linked glycosylation sites (Figure 2a). The C-terminal one-third is highly charged and is rich in proline, serine and threonine. The extreme C-terminus contains two putative nuclear localization signals, KRKKFK and RRKRSR and is hydrophilic, it is predicted to fold into a helical (possibly coiled-coil) structure (Figure 2a;

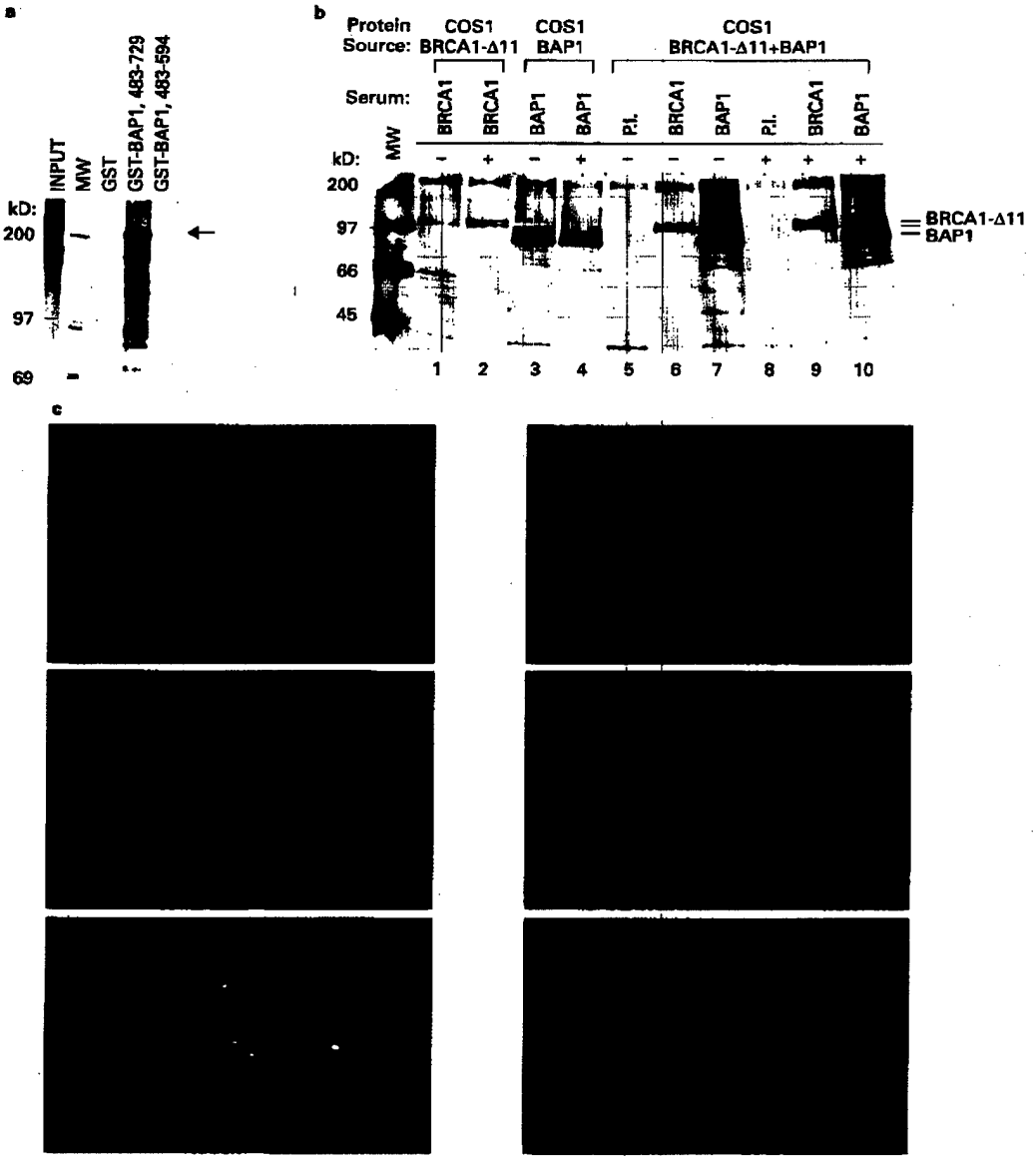

Figure 4 BAP1 and BRCA1 interact *in vitro* and *in vivo*. (**a**) BRCA1 and GST-hBAP1(483–729) interact *in vitro*. GST, GST-hBAP1(483–729) and GST-hBAP1(483–594) fusion proteins were expressed in *E. coli* and purified as described in Materials and methods. The Glutathione-Sepharose resins, containing an equivalent amount of fusion protein, were incubated in batch with *in vitro*-expressed, ^{35}S-labeled, BRCA1. After extensive washing, the proteins which remained bound were analysed by SDS–PAGE and fluorography. Lane 1, Input 2% of the labeled BRCA1 used in the associations with the glutathione resins. Lane 2, molecular weight markers. Lane 3, proteins bound to GST alone. Lane 4, proteins bound to GST-hBAP-1(483–729). Lane 5, proteins bound to GST-hBAP1-(483–594), a fusion protein lacking the BRCA1-interaction domain. Arrow indicates the BRCA1 protein. (**b**) Co-immunoprecipitation of BRCA1-Δ11 and BAP1. COS1 cells, transiently transfected with BRCA1-Δ11, BAP1, or BRCA1-Δ11 and BAP1 together, were treated with DMSO (−) or with 25 μM ALLN (+) for 16 h. Whole cell extracts were prepared after metabolic labeling and then immunoprecipitated with anti-BAP1 antibody (BAP1) or with anti-BRCA1 antibody, C20 (BRCA1) as indicated. Immunoprecipitated proteins were visualized by SDS–PAGE and fluorography. The BAP1 and BRCA1-Δ11 proteins are indicated. (**c**) BRCA1 and BAP1 partially co-localize in the nucleus. Asynchronous Rh30 cells were co-stained with the BRCA1-specific BR64 monoclonal antibody (green, top and bottom panels; Upstate Biotechnology) and a BAP1 specific, affinity-purified polyclonal antibody (red, middle and bottom panels). Top and middle panels are single color readouts of the dual color bottom panel. Yellow is produced where green and red images overlap

S Subbiah, personal communication). Indeed, within this domain the mutation of leucine 691 to a proline, a change predicted to disrupt the helical nature of this region, abolished the BAP1–BRCA1 interaction (Figure 1d). This result is consistent with the hypothesis that BAP1 uses a coiled-coil domain to interact with the RING finger domain of BRCA1. This overall architecture suggests that BAP1 is a new, structurally complex, nuclear-localized member of the UCH enzyme family.

BAP1 has UCH activity

To determine whether BAP1 did indeed have UCH enzymatic activity, BAP1 was expressed in bacteria and this protein was assayed for the ability to hydrolyze the glycine 76 ethyl ester of ubiquitin (Ub-OEt; Mayer and Wilkinson, 1989). IPTG-induced expression of BAP1 in bacteria led to abundant protein, most of which was found in an inactive, insoluble form (Figure 3a, 'BAP1 induced-Ppt'). The BAP1 protein found in the soluble

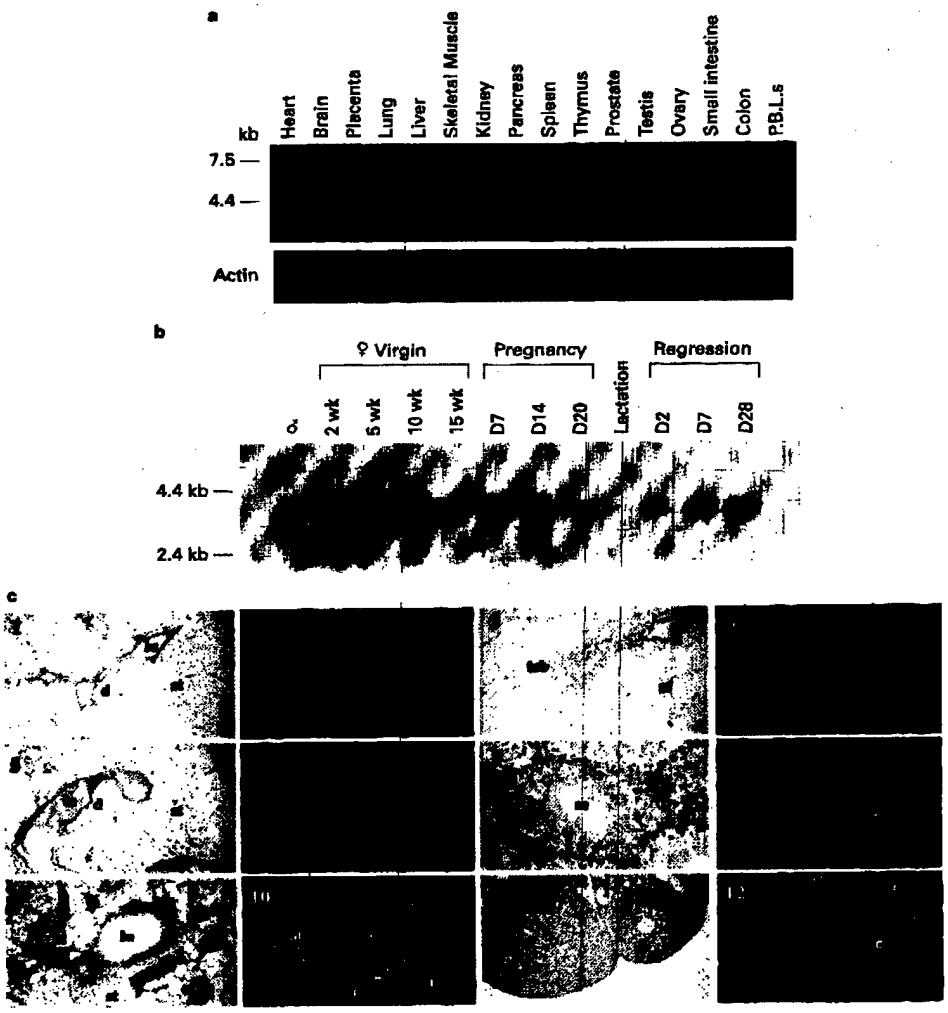

Figure 5 Tissue, spatial and temporal pattern of BAP1 expression. (a) Northern hybridization analysis of human RNA from multiple tissues. Northern blots that contain human RNA from the indicated tissues were probed with ^{32}P-labeled hBAP-1(483–729) cDNA (nts. 1488–3525). The blots were subsequently probed with a muscle actin cDNA. (b) Northern hybridization analysis of *Bap1* expression during mammary gland development. A Northern blot containing poly(A)$^+$ RNA isolated from mouse mammary glands at the indicated developmental stages was probed with ^{32}P-labeled mBAP1(596–721) cDNA. Note that mouse *Bap1* RNA is slightly smaller than the human *BAP1* RNA. (c) *In situ* hybridization analysis of *Bap1* expression. Bright-field (1, 3, 5, 7, 9 and 11) and dark-field (2, 4, 6, 8, 10 and 12) photomicrographs of *in situ* hybridization analyses performed on paraffin sections of mammary glands harvested from adolescent (1–4), mature (5, 6) or pregnant (9, 10) mice. Also shown are sections of testis (7, 8) and ovary (11, 12). To facilitate comparison, dark-field photomicrographs of breast were taken using identical shutter exposure times. al, alveolar bud; d, ductal epithelium; lu, ductal lumen; st, stroma; teb, terminal end bud; d, bm, basement membrane; se, semeniferous tubule; f, developing follicle; g, granulosa cells; th, thecal cells; ct, connective tissue

fraction was able to hydrolyze Ub-OEt, indicating that BAP1 contains UCH-like enzymatic activity (Figure 3b). The active site thiol residue responsible for UCH activity in UCH-L3 has been identified and its mutation leads to abolition of enzyme activity (Larsen *et al.*, 1996). Mutation of the corresponding cysteine residue in BAP1, BAP1(C91S), yielded a protein with no detectable UCH activity (Figure 3b) further supporting the conclusion that BAP1 is a thiol protease of the UCH family.

BAP1 associates with BRCA1 in vitro *and* in vivo

Association of BRCA1 with BAP1 was tested *in vitro* by binding of full-length BRCA1 to hBAP1(483–729) fused to glutathione S-transferase (GST; Figure 4a). The ^{35}S-labeled BRCA1, produced by coupled *in vitro* transcription and translation, specifically bound to the GST–hBAP1(483–729) fusion protein, but not to GST alone (Figure 4a) indicating a physical association between the two proteins. As predicted from the yeast two-hybrid results (Figures 1d and 4a), BRCA1 did not bind to GST–hBAP1(483–594), a GST–BAP1 fusion protein lacking the BRCA1 interaction domain.

To determine whether BRCA1 and BAP1 could interact in mammalian cells, a co-immunoprecipitation analysis from co-transfected COS1 cells was performed (Figure 4b). Several attempts to transiently express BRCA1 to any significant level in COS1 cells were without success. Therefore, we performed the analysis with BRCA1-Δ11, a naturally occurring splice variant (Thakur *et al.*, 1997) which can be expressed in these cells and which contains the RING finger domain. The proteasome/caspase inhibitor ALLN (N-acetyl-L-Leucinyl-L-Leucinyl-L-norLeucinal) was included in the analysis to determine its influence on the stability of the interaction between BRCA1 and BAP1. BRCA1-Δ11 was detected by immunoprecipitation as a sharp band at ~99 kDa in singly transfected COS1 cells (Figure 4b, lane 1). Incubation of a parallel set of transfected cells with ALLN (20 h) prior to harvest revealed a discrete slower migrating band in the anti-BRCA1 immunoprecipitates (Figure 4b, lane 2). Immunoprecipitates from BAP1-transfected COS1 cells revealed a 91 kDa protein whose mobility was apparently unaffected by treatment with ALLN (Figure 4b, lanes 3 and 4). Co-transfection of COS1 cells with BRCA1-Δ11 and BAP1 revealed that these proteins could be co-immunoprecipitated using either anti-BAP1 or anti-BRCA1 antibodies (Figure 4b, lanes 9 and 10); the ability to detect the BRCA1–BAP1 complex under these conditions was dependent upon incubation of the cells with the proteasome inhibitor. In co-transfected, ALLN-treated cells, both forms of BRCA1-Δ11 are evident in the co-immunoprecipitated complex, and both forms are more abundant than singly transfected cells (compare lanes 2 and 10). These results demonstrate the *in vivo* association of BRCA1 and BAP1, and suggest the presence of a proteasome inhibitor-sensitive modification of BRCA1 which may enhance its interaction with BAP1.

To further document the *in vivo* interaction between BAP1 and full-length BRCA1, we determined whether the endogenous (non-transfected) proteins were co-localized in the cell nucleus (Figure 4c). A mouse monoclonal antibody to BRCA1 (Maul *et al.*, manuscript in preparation) and affinity purified rabbit BAP1 antibody were used to stain rhabdomyosarcoma cells (Rh30), a cell line previously determined to express *BAP1* RNA (Jensen and Rauscher, unpublished results). BRCA1 was detected exclusively in punctate domains within the nucleus (excluding nucleoli) in agreement with other reports (Jin *et al.*, 1997; Scully *et al.*, 1997b). BAP1 was also detected in punctate domains within the nucleus of Rh30 cells, however, the number of domains was significantly greater than for BRCA1. Several of the BRCA1 domains coincided with BAP1 domains (yellow dots in the bottom panels), suggesting an endogenous BRCA1-BAP1 interaction. We also detected BRCA1 reactive domains which were not co-localized with BAP1 domains as has been seen with other BRCA1-interacting proteins (Figure 4c; Jin *et al.*, 1997; Scully *et al.*, 1997b). These data show that BAP1 and BRCA1 can physically interact *in vitro* and *in vivo*, and have overlapping subnuclear expression patterns.

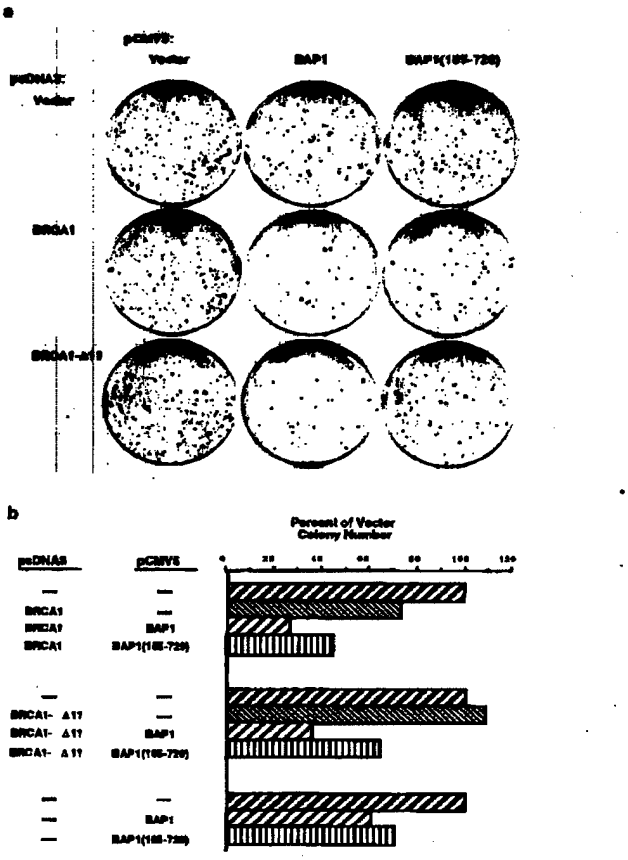

Figure 6 BAP1 enhances BRCA1-mediated growth suppression. (**a**) MCF7 cells were co-transfected with each of the plasmid constructs shown. Cells were then harvested and 5×10^5 cells were plated in duplicate into complete medium containing G418. Twenty-one to 28 days later, cells were stained and colonies counted. The experiment was repeated four times with similar results. (**b**) Quantitation of the results from (**a**)

BAP1 is expressed in a temporal and spatial pattern during breast development/remodeling

The physical interaction between BAP1 and BRCA1 suggests that the proteins might be expressed in similar tissues. Northern blot hybridization analysis of *BAP1* expression in a variety of human adult tissues indicated that human *BAP1* was encoded by a single mRNA species of ~4 kb in all tissues except testis, where a second, ~4.8 kb mRNA, was also detected (Figure 5a). High expression was detected in testis, placenta and ovary, with varying levels detected in the remaining tissues. Expression of *BAP1* in normal human breast tissue was detected by RT–PCR of total RNA isolated from normal human mammary epithelial cells (data not shown).

Northern analysis and *in situ* hybridization were performed to determine whether the spatial and temporal pattern of *Bap1* expression in the breast corresponded to that previously described for *Brca1* (Figure 5b and c; Marquis *et al.*, 1995). *Bap1* was expressed at slightly higher levels in the mammary glands of 5 week-old adolescent female mice compared to 15 week-old mature mice. Consistent with this, *in situ* hybridization revealed high level of *Bap1* mRNA expression in terminal end buds (Figure 5c), and higher levels of *Bap1* expression in the ductal epithelium of adolescent as compared with mature female mice. Like *Brca1*, *Bap1* mRNA was expressed in the mammary epithelium at levels higher than those found in the stromal compartment. Steady-state levels of *Bap1* mRNA were up-regulated in the mammary glands of pregnant mice (Figure 5b and c). Like *Brca1*, *in situ* hybridization demonstrated that this up-regulation occurred predominantly in developing alveoli (Figure 5c). *Bap1* was expressed at higher levels in the mammary glands of parous animals that had undergone 28 days of post-lactational regression as compared with age-matched virgin controls (cf. D28 regression and 15 week virgin). The observation that *Bap1* expression is up-regulated in the breast during puberty, pregnancy and as a result of parity is similar to that previously made for *Brca1* and suggests the developmental co-expression of these two proteins (Marquis *et al.*, 1995). It should be noted, however, that the magnitude of *Bap1* up-regulation at each of these developmental stages was lower than that observed for *Brca1*. The spatial distribution of *Bap1* expression was also investigated in the testis and ovary (Figure 5c). Like *Brca1*, *Bap1* was expressed at high levels in semeniferous tubules, with lower levels of expression observed in the surrounding connective tissue. *Bap1* was also expressed at high levels throughout the ovary.

BAP1 augments the growth suppressive activity of BRCA1

Several studies have shown that BRCA1 can affect the growth characteristics of cells (Holt *et al.*, 1996; Rao *et al.*, 1996). We determined whether BAP1 itself may affect cell growth or may alter BRCA1-mediated changes in cell growth. *BRCA1* and *BAP1* cDNAs were co-transfected into MCF7 breast cancer cells and analysed for their effect on colony formation (Figure 6). This cell line was chosen for several reasons: First,

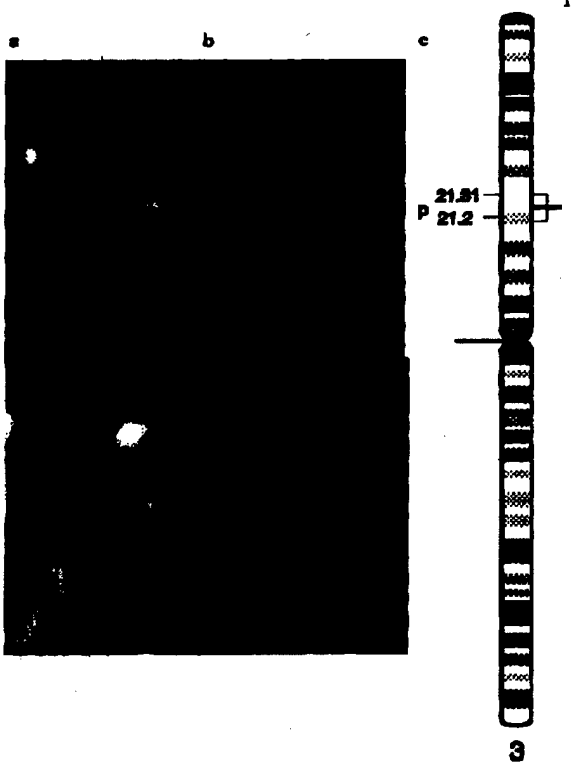

Figure 7 *BAP1* maps to Chromosome 3p21.3. Fluorescence *in situ* hybridization (FISH) of partial metaphases using biotin-labeled BAP1 cDNA. (a) the specific FISH signals on chromosome 3 (arrows), with (b) the simultaneously DAPI-stained chromosomes and (c) a chromosome ideogram with the localization of BAP1 based on the DAPI-band pattern and FLpter value. The horizontal box indicates the variation in FLpter values on individual chromosomes

it previously has been shown that the growth of these cells is inhibited by the overexpression of *BRCA1* (Holt *et al.*, 1996). Second, both Northern and RT–PCR analyses showed that BAP1 is expressed in this cell line (data not shown). Finally, RT–PCR/SSCP analysis of the open reading frame of *BAP1* cDNA prepared from this cell line showed no mutations (data not shown).

The expression of BRCA1 alone (BRCA:pCMV5) decreased the number of colonies formed by these cells when compared to the vector control (pcDNA3:pCMV5), in agreement with other studies (Holt *et al.*, 1996). The co-expression of BRCA1 and BAP1 (BRCA1:BAP1) significantly decreased the number of cell colonies (approximately fourfold *vs* BRCA1 alone; see Figure 6b) indicating that BAP1 enhances the growth suppressive actions of BRCA1. A mutant of BAP1, BAP1(165–729), in which the enzymatic region is deleted but which still binds to BRCA1, also enhanced the growth suppression of BRCA1, but not to the same extent as the wild-type BAP1.

The expression of BRCA1-Δ11 (a naturally occurring splice variant of BRCA1) in MCF7 cells by itself had no effect on the growth of MCF7 cells (Figure 6).

However, the co-expression of BRCA1-Δ11 and BAP1 dramatically decreased the number of colonies, suggesting that the presence of BAP1 could cooperate with BRCA1-Δ11 in cell growth inhibition. Furthermore, the expression of BAP1 in MCF7 cells also reduced the number of colonies formed (pcDNA3:BAP1; see Figure 6b). The expression of the enzymatic mutant, BAP1(165–729), alone or in combination with BRCA1-Δ11, yielded no growth-suppression activity. Thus, enzymatically active BAP1 enhances BRCA1-mediated suppression of growth in this assay.

BAP1 is located on chromosome 3p21.3 and is mutated in non-small cell lung carcinoma

The data above suggest the possibility that *BAP1* itself may be a tumor suppressor gene and that *BAP1* alterations/deletions might play a role in human tumorigenesis. We determined whether *BAP1* might be located at a chromosomal region previously observed to be mutated in human cancers. The full-length *BAP1* cDNA was used in fluorescent *in situ* hybridization (FISH) to identify the chromosomal location of the *BAP1* gene (Figure 7). Specific signals were observed only on the midportion of the short arm of chromosome 3 with 42 of 69 analysed metaphase spreads showing at least one specific signal. The FLpter value was 0.27 ± 0.02 corresponding to a localization for *BAP1* at 3p21.2-p21.31. This location is a region of LOH for breast cancer as well as a region frequently deleted in lung carcinomas (Buchhagen *et al*., 1994; Thiberville *et al*., 1995).

The chromosomal location of *BAP1* suggested the possibility of mutations within *BAP1* in lung tumors. Thus, a total of 44 small cell lung cancer (SCLC), 33 non-small cell lung cancer (NSCLC) and two lymphoblastoid tumor cell lines were screened for mutations within the *BAP1* gene by Southern, Northern and PCR-based SSCP analyses. Genomic DNAs from 15 small cell lung cancer (SCLC), 18 non-small cell lung cancer (NSCLC) and two lymphoblastoid cell lines (representing a subset of the total), were subjected to *Eco*RI digestion and then hybridized to a full-length

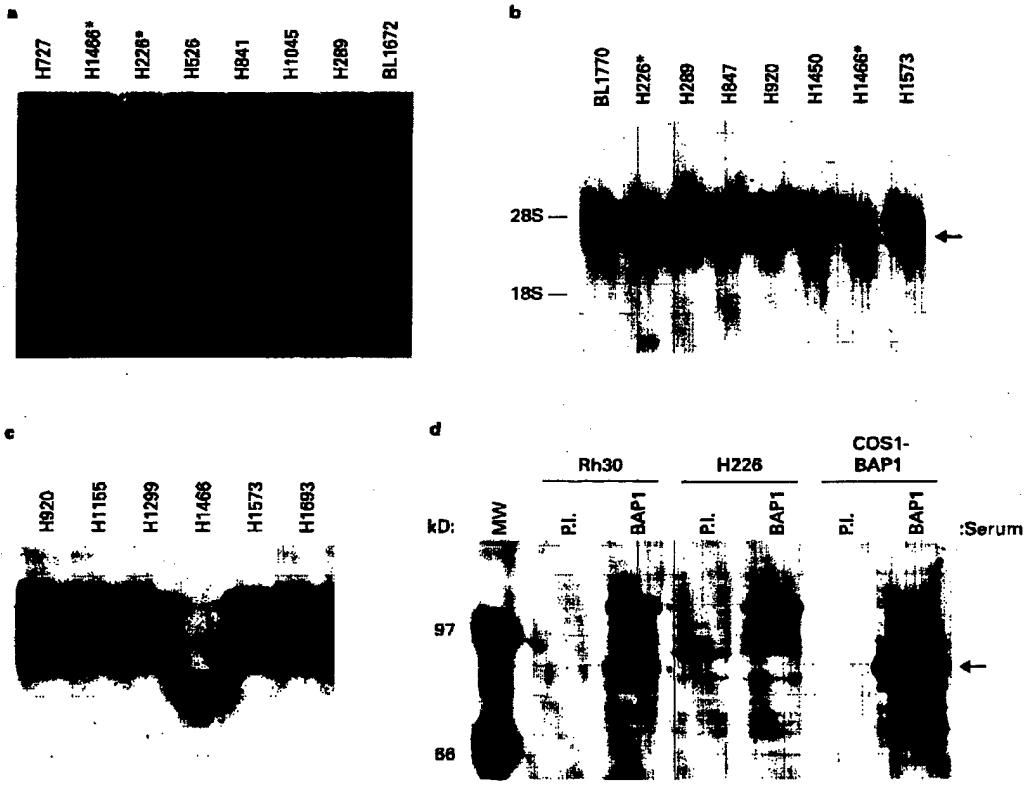

Figure 8 Mutational analysis of lung carcinomas. (**a**) Southern blot hybridization with *Bam*HI digestion showing four distinct bands at 7.5 kbp, 4.0 kbp, 3.0 kbp and 2.4 kbp as detected by a full-length BAP1 probe. The non-small cell lung cancer NCI-H226 line shows an absence of the 7.5 kbp, 4.0 kbp and 3.0 kbp bands. An aberrant 2.6 kbp band is detected in the NCI-H226 cell line. (**b**) Northern blot hybridization showing a ~4 kb message (arrow) which is absent in the NCI-H226 cell line and the non-small cell lung cancer NCI-H1466 cell line. A fainter (5.0 kb) band is visible corresponding to cross hybridization with the 28S ribosomal component. (**c**) SSCP analysis showing a homozygous shift in H1466 detected by RT–PCR amplification spanning nts 1089 to 1286. (**d**) BAP1 is a 90 kD protein and is missing from NCI-H226 cells. Endogenous BAP1 from Rh30 or H226 cells, or BAP1 expressed in COS1 cells from the BAP1 cDNA, was immunoprecipitated from whole cell lysates prepared after ^{35}S-labeling of 1×10^7 cells. Immunoprecipitated proteins were analysed by SDS–PAGE followed by fluorography. The arrow indicates the BAP1 protein. All cell lines in panels **a**, **b** and **c** are listed by their NCI number (Phelps *et al*., 1996)

BAP1 cDNA probe. A single 23 kb band was detected in the lymphoblastoid and most tumor cell lines (data not shown). One NSCLC line, NCI-H226, did not show the 23 kbp band but did show an aberrant 30 kbp band (data not shown). This abnormality was confirmed by *Bam*HI, *Xba*I, *Pst*I and *Bgl*II digestions. Using the 3.5 kbp BAP1 cDNA probe with *Bam*HI digested genomic DNAs, we detected four distinct bands at 7.5 kbp, 4.0 kbp, 3.0 kbp and 2.4 kbp which were present in all cell lines tested with the exception of NCI-H226 (a representative subset is shown in Figure 8a). In the NCI-H226 line, we detected only the 2.4 kbp band and an aberrant 2.6 kbp band.

Further mutational analysis of BAP1 was performed by screening a subset of 31 SCLC and 27 NSCLC lung cancer cell lines and two lymphoblastoid cell lines for expression of *BAP1* mRNA. Northern analysis showed that most cell lines expressed a single ~4 kb mRNA (Figure 8b). However, two cell lines, NCI-H226 and NCI-H1466 (both NSCLCs), showed undetectable levels of *BAP1* expression, suggesting that *BAP1* may be a target for inactivation or down-regulation during NSCLC pathogenesis. The absence of BAP1 protein in the NCI-H226 line was determined by immunoprecipitation of BAP1 from ^{35}S-labeled cells (Figure 8d).

We screened cDNAs from all 44 SCLC and 33 NSCLC cell lines for mutations in the BAP1 open reading frame by RTPCR-SSCP (Figure 8c). A homozygous, eight base pair deletion and a presumed splice variant were detected. An 8 bp deletion was detected in the cDNA from the NSCLC line NCI-H1466; This short deletion leads to a frameshift/truncation yielding a predicted 393 amino acid protein. This homozygous deletion was confirmed in genomic DNA from the same cell line (data not shown). A 54 bp in-frame deletion (nts. 705-758) was detected in the NCI-H1963 (SCLC) cell line. This deletion was heterozygous at the cDNA level and not present in the genomic DNA, suggesting that it is a splice variant. However, this variant was not detected in any of the other cell lines screened. These data clearly show that genetic alterations, including intragenic homozygous deletions, can occur in *BAP1*.

Discussion

We have discovered and characterized a novel protein, BAP1, which binds to the BRCA1 RING finger motif. Several lines of evidence are offered which support a role for BAP1 in BRCA1 signal transduction pathways. We showed that: (1) BAP1 binds to the RING finger of BRCA1; but not to germline mutants of BRCA1 or related RING domains; (2) The BAP1--BRCA1 interactions occurs *in vitro* and *in vivo*, and these proteins are partially co-localized in nuclear dot structures; (3) *BAP1* mRNA is expressed in those tissues which also expresses *BRCA1*, and the spatial/temporal distribution of *Bap1* expression in the mouse breast is very similar to that observed with *Brca1*; (4) BAP1 enhances BRCA1-mediated suppression of cell growth in colony formation assays, and suppression by BAP1 is augmented by its UCH enzymatic domain; and (5) *BAP1* maps to chromosome 3p21.3 and is homozygously deleted in a lung carcinoma cell line. Together, these observations suggest that *BAP1* may be a tumor suppressor gene, and that it may serve as a regulator of (or is an effector for) BRCA1 growth control/differentiation pathways. The specificity of the BRCA1 RING finger-BAP1 interaction and the fact that independent, germline missense mutations in the BRCA1 RING finger domain abolish interaction with BAP1 provide substantial evidence for the physiological relevance of this interaction.

BAP1 is a nuclear-localized, ubiquitin carboxy-terminal hydrolase (UCH) which can cleave model ubiquitin substrates *in vitro*. The UCH homology of BAP1 implies a role for either ubiquitin-mediated, proteasome-dependent degradation or other ubiquitin-mediated regulatory (Isaksson *et al.*, 1996) pathways in BRCA1 function. Regulated ubiquitination of proteins and subsequent proteasome-dependent proteolysis plays a role in almost every cellular growth, differentiation and homeostatic process (reviewed by Ciechanover, 1994; Isaksson *et al.*, 1996; Wilkinson, 1995). The pathway is regulated both at the level of substrate specificity – via the concerted actions of activating enzymes, carrier proteins and ligation enzymes – and at the level of proteolytic deuibiquitation and ubiquitin hydrolysis. The latter enzymes are ubiquitin-specific thiol proteases which have been broadly classified into two families: the ubiquitin-specific protease (UBPs) and the ubiquitin carboxy-terminal hydrolases (UCHs).

The UBP family members are 50-300 kDa, cytoplasmic or nuclear-localized proteins which, in general, cleave ubiquitin or ubiquitin-conjugates from large substrates. Such enzymatic activity can be found directly associated with the 26S proteasome and may serve a regulatory function by editing ubiquitin on large substrates or cleaving polyubiquitin, thus replenishing ubiquitin pools (Lam *et al.*, 1997). Remarkably, a number of UBPs have been isolated as growth regulatory and/or developmental control genes such as DOA4 in yeast, which controls DNA replication and repair (Papa and Hochstrasser, 1993); UBP3 which is involved in transcriptional silencing in yeast (Moazed and Johnson, 1996); the TRE2 oncogene which is mutated in the UBP active site and functions as a dominant negative transforming gene (Nakamura *et al.*, 1992); the drosophila *Fat Facets* gene which controls pattern formation and eye development (Huang *et al.*, 1995; Huang and Fischer-Vize, 1996); and the human DUB family of cytokine-inducible UBPs which control hematopoietic differentiation (Zhu *et al.*, 1996, 1997).

By contrast, the UCH family has been characterized as a set of small (25-30 kDa) cytoplasmic proteins which prefer to cleave ubiquitin from ubiquitin-conjugated small substrates and may also be involved in the co-translational processing of proubiquitin. Like the UBPs, UCHs show considerable tissue specificity and developmentally-timed regulation (Wilkinson *et al.*, 1992). UCH family members are differentially expressed in neuronal, hematopoietic and germ cells in many species. Most remarkably, a novel UCH enzyme has recently been cloned from *A. californica* whose enzymatic function is essential for acquisition and maintenance of long-term memory (Hedge *et al.*, 1997). Finally, UCH levels are down-regulated during viral transformation of fibroblasts (Honore *et al.*, 1991), consistent with a role in growth control.

BAP1 is the newest member of the UCH family and considerably expands the potential roles of this family of proteases. BAP1 is a much larger protein (90 kDa) and is the first nuclear-localized UCH. In addition to containing the ~250 amino acid amino-terminal UCH catalytic domain, it includes a carboxy-terminal extension rich in proline, serine and threonine, and a short, highly acidic region; these elements may confer a short half-life upon the protein (Rechsteiner and Rogers, 1996). The extreme carboxy-terminus encodes two potential nuclear localization signals that overlap the approximately 125 amino acid BRCA1-interaction domain. This domain is predicted to fold into a long amphiphatic helix of coiled-coil character, the structure of which may be important for BRCA1 interaction. Indeed, truncation into this region or substitution of a proline for leucine (L691P) abolish the BAP1–BRCA1 interaction in the two-hybrid assay. We have also detected a potential splice variant in BAP1 that results in loss of 31 amino acids of the BRCA1 interaction domain and greatly reduces the ability of BAP1 to bind the BRCA1 RING finger. Thus, our data suggest that the BAP1 carboxy-terminus is tethered to the BRCA1 RING finger domain, leaving the UCH catalytic domain free to interact with ubiquitinated, or other ubiquitin-like, substrates.

A simple model explaining most of our data is that BRCA1 is a direct substrate for the UCH activity of BAP1 and deubiquitination results in the stabilization of BRCA1. Thus, in contrast to the known UCHs which are comprised entirely of the UCH domain, the carboxy-terminal extension of BAP1 may provide substrate and/or targeting specificity for the catalytic function. Paradigms for separate substrate recognition and catalytic domains occur throughout the ubiquitin conjugation/ligation system (see Wilkinson, 1995 and references therein). Regulated ubiquitination of BRCA1 and subsequent proteasome-mediated degradation would be consistent with tight regulation of BRCA1 levels and its subnuclear localization during both the mitotic cell-cycle and meiosis (Gudas *et al*., 1996; Scully *et al*., 1997b; Zabludoff *et al*., 1996). Thus, BAP1-mediated deubiquitination of BRCA1 would stabilize the protein and protect it from proteasome-mediated degradation. This scenario is consistent with both the ability of co-transfected BAP1 to enhance the tumor suppressor effects of BRCA1 in colony formation assays and the finding of mutations in BAP1 in cancer cell lines.

A second, and equally plausible hypothesis is that the BRCA1–BAP1 association serves to target the UCH domain to other substrates that may be bound to other sites on BRCA1. In this scenario, BRCA1 may function as an assembly or scaffold molecule for regulated assembly of multiprotein complexes; a function that has been postulated for other tumor suppressor proteins (e.g. pRb; Sellers and Kaelin, 1996; Welch and Wang, 1995). Thus, BAP1 could be a regulator of this assembly via its control of ubiquitin-mediated proteolysis. Recently, it has been shown that the RING finger protein Ste5 (*S. cerevisiae*) functions as a scaffold protein for assembling protein kinase-dependent signaling complexes in pheromone signaling; this activity is abolished by mutations in the Ste5 RING finger (Inouye *et al*., 1997). In this context, two other RING finger-containing proteins are involved in complexes whereby controlled proteolytic processes are dependent upon the integrity of the RING finger structure: (1) The murine homologue of the drosophila *seven-in-absentia*, siah, (a RING finger protein) binds to the tumor suppressor protein Deleted in Colon Cancer (DCC) and targets it for proteasome-mediated degradation. This degradation requires the siah RING finger structure (Hu *et al*., 1997); and (2) The herpesvirus protein VMW110 is a RING finger protein that binds directly to a UBP family member, HAUSP, and appears to target it to the ND10/POD nuclear dot structure. The ND10 structure itself contains another RING finger protein, the proto-oncogene PML (Everett *et al*., 1997). Remarkably, the PML RING finger has been shown to bind and co-localize with a ubiquitin-like molecule PIC1/SUMO1 which is emerging as a central molecule in nuclear-localized ubiquitin-dependent regulation (Saitoh *et al*., 1997).

A third hypothesis is that BAP1 is involved in the regulation of protein subcellular localization. Mono-ubiquitination, or addition of a ubiquitin-like moiety, has emerged as an important post-translational modification which may affect the specific targeting of proteins to locations other than the proteasome. For example, the addition of the ubiquitin-like PIC1/SUMO1 protein appears to mediate the movement of RanGAP1 (a GTPase-activating protein) from the cytoplasm to the nuclear envelope where it binds the RanBP2 protein (Mahajan *et al*., 1997). This interaction requires the ATP-dependent, covalent addition of PIC1/SUMO-1 to RanGAP1, a process extremely similar to the ATP-dependent, ubiquitin-ligation mechanism. Thus, BAP1-mediated removal of ubiquitin, or ubiquitin-like molecules, from BRCA1, or a protein associated with BRCA1, could target the complex to another cellular compartment, thus altering its function without physically destroying it. That BRCA1 is targeted to specific subcellular sites is evidenced by the observation that it accumulates in nuclear dot structures during S phase of the cell cycle. This localization is abolished at the S/G2 boundary (Scully *et al*., 1997b).

The association of BRCA1 with RAD51 in both mitotic nuclear dot structures and meiotic cells broadly implicates BRCA1 in DNA repair and/or recombination processes. The RAD51/52-dependent DNA repair pathway is highly regulated and includes many proteins, some of which may be potential substrates for BAP1-mediated ubiquitin hydrolysis. RAD23, which associates with the RAD51/52 complex, contains an amino-terminal ubiquitin-like domain which is required for RAD23 function and double-strand break repair (Watkins *et al*., 1993). Recently, a human ubiquitin-like protein, UBL-1, was isolated as a protein which binds directly to the human RAD51/RAD52 complex (Shen *et al*., 1996b). Interestingly, the yeast homologue of UBL1 is SMT3, which functionally associates with the yeast centromere protein MIF2, a protein required for proper chromosome segregation (Brown, 1995; Brown *et al*., 1993). Furthermore, the RAD51/52 complex contains a ubiquitin conjugating enzyme, hUBC9/UBE2I (Jiang and Koltin, 1996; Shen *et al*., 1996a). Thus, it appears that the DNA repair machinery contains both ubiquitin-conjugating and -hydrolyzing

elements, since BAP1 is now implicated as a member of the BRCA1/RAD51/hUBC9 complex. It is possible that BAP1, which is co-expressed with BRCA1 in breast tissue, may regulate the recombination/repair functions of the BRCA1/RAD52 complex by targeting either RAD23 or UBL1 for ubiquitin hydrolysis.

The implications that BAP1 is a key regulator and/or effector of BRCA1 suggest that BAP1 may also play a role in human cancer. The finding that *BAP1* maps to human chromosome 3p21.3 strongly suggested this link; Loss of chromosome 3p genes is a critical event in lung cancer pathogenesis and other carcinomas. Interestingly, two other components of the ubiquitin metabolism pathway have been mapped to chromosome 3p21.3; an ubiquitin activating enzyme (Kok *et al.*, 1993) and a ubiquitin protease, UNP (Gray *et al.*, 1995). The identification of BAP1 as a UCH suggests a cluster of metabolically related enzymes at this locus. Furthermore, the frequent loss of this chromosomal region suggests that there may be a selective advantage for the loss of ubiquitin-mediated cellular processes during carcinogenesis. That BAP1 may be included in this paradigm is suggested by our detection of rearrangements/deletions within BAP1 in lung cancers (this report) and our detection of independent, homozygous, point mutations in highly conserved residues of BAP1's enzymatic domain (Proctor *et al.*, manuscript in preparation).

In summary, we have isolated a novel ubiquitin hydrolase which associates directly with the BRCA1 RING finger domain. BAP1 may play a key role in ubiquitin-dependent regulatory processes in the nucleus including transcription, chromatin remodeling, cell cycle control and DNA repair/recombination.

Materials and methods

Cell culture, transfections and colony formation assays

All cells were grown at 37°C and 5% CO_2. COS1 and MCF7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine and non-essential amino acids. Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cell lines were maintained in RPMI media with 10% fetal bovine serum (Gibco BRL). Rh30 cells were maintained in RPMI supplemented with 10% FBS and non-essential amino acids. COS1 cells were transfected using DOSPOR transfection reagent (Boehringer Mannheim Biochemicals) following the manufacturer's protocol.

Colony formation assay

MCF7 cells were transfected by a modified $CaPO_4$-DNA precipitation method (Holt *et al.*, 1996). MCF7 cells, at 2×10^6 cells/10 cm dish, were fed fresh medium approximately 3 h prior to transfection, and were then treated with the Ca-DNA precipitate for 4 h. The cells were subjected to a brief shock with transfection buffer containing 15% glycerol. Twelve to 16 h later, the cells were trypsinized, counted and plated directly into complete medium containing 0.75 mg/mL G418 at 5×10^5 cells per 10 cm dish. Cells were fed fresh medium containing G418 every 3–4 days. Cells were stained for colonies approximately 21–28 days after transfection.

Yeast 2-hybrid

The yeast 2-hybrid system as modified by Stan Hollenberg was used for all yeast experiments (Vojtek *et al.*, 1993). Two libraries were screened for interaction with LexA-BRCA1; a human B cell, oligo-dT-primed, cDNA library (Durfee *et al.*, 1993); a kind gift from Dr Steve Elledge) and a mouse embryo (9.5–10.5 day), random-primed, cDNA library size selected for inserts of 300–500 base pairs in length (Vojtek *et al.*, 1993); a kind gift of Dr Stan Hollenberg).

Construction of expression plasmids

LexA fusion constructs: The 100 amino acid terminal region of human BRCA1 (BRCA1-RF) was used as the bait to screen for interacting proteins. All LexA fusion constructs were made by cloning the appropriate RING (or other) domain into the vector pBTM-116 (Vojtek *et al.*, 1993). A synthetic gene of the BRCA1–RF domain was made from overlapping oligonucleotides whose codon usage had been optimized for expression in *E. coli* and *S cerevisiae* (Madden *et al.*, 1991). Double-stranded DNA was generated by the Polymerase Chain Reaction (PCR) and amplified with flanking primers containing *Eco*RI and *Sal*I enzymatic restriction sites. A 'wild type' BRCA1–RF domain was confirmed by DNA sequencing. The single amino acid substitutions in the BRCA1–RF domain, BRCA1(C64G) (Cys 64 to Gly), BRCA1(C61G) (Cys 61 to Gly), BRCA1(M18T) (Met18Thr) and BRCA1(R71G) (Arg71 to Gly) as well as the BRCA1–delAG185 truncation mutant were created by PCR-mutagenesis (Ho *et al.*, 1989). The BRCA1-del31 truncation mutant was a mis-primed PCR reaction of the BRCA1–RF which was identified by DNA sequencing. The LexA-RPT-1 protein (amino acids 1–100) was made by PCR-mediated amplification of the corresponding nucleotides of a RPT-1 PCR sample (Patarca *et al.*, 1988); kindly provided by Dr Harvey Cantor). All clones were confirmed by sequencing. Expression of all constructs in yeast was confirmed by Western analysis using antibodies against the LexA DNA-binding domain (data not shown).

BAP1 constructs: A full-length BAP1 cDNA was assembled through the fusion of two overlapping EST clones (the IMAGE Consortium (LLNL); cDNA Clones #46154 and #40642; (Lennon *et al.*, 1996)) and the insertion of 62 nucleotides missing from clone #40642 (as revealed by sequencing and RT–PCR analyses). GST-hBAP1(483–729) was generated by cloning the *Xho*I fragment of pAct (the original human two-hybrid clone; nucleotides 1486–3525) into pGEX-5x-1 (Pharmacia Biotech, Inc.). GST-hBAP1(438–594) and pACT-hBAP1(438–594) (nucleotides 1486–1821) were generated and amplified by PCR, digested with restriction enzyme and ligated into the appropriate vector.

Mapping of BRCA1/BAP1 interaction domain

Truncations of mBAP(596–721), and the point mutation mBAP1(L691P), were generated by PCR-based mutagenesis. Products were then ligated into the mouse library-yeast expression vector, pVP16. All clones were confirmed by sequencing and expression in yeast was confirmed by Western analysis using antibodies against the VP16 activation domain (data not shown).

Northerns and in situ

Tissue RNA blots were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Blots were hybridized with ^{32}P-labeled hBAP1(483–729) cDNA (nucleotides 1486–3525) using standard protocols. RNA

preparation, Northern hybridization of mouse mammary tissue, and *in situ* hybridization analyses were performed as previously described using the mouse Bap1 cDNA probe described by mBAP1(596–721), corresponding to nucleotides 1825–2202 of the human probe (Marquis *et al.*, 1995).

Fluorescent in situ *hybridization (FISH)*

FISH using a biotin-labeled 3.5 kb cDNA (full length) clone of BAP1, with corresponding DAPI-banding and measurement of the relative distance from the short arm telomere to the signals (FLpter value) was performed as described previously (Tommerup and Vissing, 1995).

Immunolocalization

All immunofluorescence was performed as previously described (Ishov and Maul, 1996). BAP1 protein was detected with affinity purified, BAP1-specific polyclonal antibodies, and BRCA1 was detected with the BR64 monoclonal antibody (Upstate Biotechnology), which were detected with FITC and Texas Red (respectively) using biotin-avidin enhancement. Cells were stained for DNA with bis-benzimide (Hoescht 33258, Sigma Chemical Co.) and mounted using Fluoromount G (Fisher Scientific). Analysis was performed with a confocal scanning microscope (Leica, Inc.).

BAP1 protein characterization

Generation of antibodies: Using PCR cloning, the cDNA region encoding amino acids 483–576 of BAP1 was fused downstream of the six Histidine residues of the vector pQE-30 (QIAGEN Inc.). The His-tagged protein was purified from *E. coli* over a Ni-agarose column as previously described (Friedman *et al.*, 1996) and was used to immunize rabbits for the production of polyclonal antibodies (Cocalico Biologicals, Inc.). Immunoprecipitation of BAP1 was performed by previously described procedures for the metabolic labeling and immunoprecipitation of proteins from cell lysates (Friedman *et al.*, 1996).

In vitro *protein association:* GST, GST-hBAP1(483–729) and GST-hBAP1(483–594) were expressed in *E. coli* and then purified as described (Frangioni and Neel, 1993). The ^{35}S-BRCA1 protein was produced *in vitro* via coupled transcription/translation (TNT®, Promega Corp.). Association between proteins was assayed as described previously (Barlev *et al.*, 1995).

BAP1 enzymatic assay

Assays for BAP1 enzymatic activity were performed essentially as described for the UCH-L1 and UCH-L3 enzymes (Mayer and Wilkinson, 1989). Briefly, bacteria harboring an IPTG-inducible expression plasmid containing BAP1 (pQE-30; QIAGEN Inc.) were grown and induced with 1 mM IPTG for 4 h. The bacteria were collected and the pellets were resuspended to 1/20 volume (original culture) in lysate buffer (50 mM Tris, pH 8.0, 25 mM EDTA, 10 mM 2-mercapto-ethanol, 100 μg/ml lysozyme). The lysates were sonicated and centrifuged at 40 000 *g*. The soluble fractions were used for subsequent activity assays. The pellets were resuspended in a volume equal to that of the supernatant and samples of both pellet and supernatant were analysed by SDS–PAGE for expression levels and inclusion body formation.

Assays for ubiquitin carboxy-terminal hydrolase activity were performed using the glycine 76 ethyl ester of ubiquitin (Ub-OEt) as substrate (Mayer and Wilkinson, 1989; Wilkinson *et al.*, 1986). Assays were done in triplicate. The peak areas were integrated and normalized with respect to a ubiquitin standard.

Mutation screening

RNA/DNA preparation: Genomic DNA was prepared from breast and lung cancer cell lines using standard methods. Total RNA was extracted by the cesium chloride-ultracentrifugation method (Ausubel *et al.*, 1987). First strand cDNAs were synthesized from RNA by M-MLV reverse transcriptase (Gibco–BRL) according to the manufacturer's instructions.

Southern and Northern blot hybridization: Five μg of genomic DNA, subjected to restriction enzyme digestion, or 10 μg total RNA, was electrophoretically gel-fractionated and transferred to Hybond N$^-$ membranes (Amersham). Hybridization was performed with a ^{32}P-full-length BAP1 cDNA probe followed by washes under standard conditions and detection by autoradiography.

Single strand conformational polymorphism (SSCP) analysis: Seventeen overlapping PCR primer pairs, each with a predicted product size of approximately 200 base pairs, were designed to span the 2.2 kb open reading frame of the BAP1 cDNA sequence. cDNA (from RNA) was amplified in 20 μl PCR reactions containing 20 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl, 0.2 mM each dNTP, 0.1 mM each forward and reverse primer, 0.05 ml [^{32}P-α]dCTP and 0.5 units Taq DNA Polymerase (BRL). PCR reactions were carried out in a Perkin-Elmer 9600 Thermocycler using a touchdown technique: a 2.5 min initial denaturation at 94°C was followed by 35 cycles of denaturation at 94°C × 30 s, annealing, initially at 65°C decreasing by 1°C for each of the first ten cycles to 55°C, × 30 s, and extension at 72°C × 30 s with a final extension of 5 min at 72°C. PCR products were then diluted 1:10 with SSCP dye (95% formamide, 20 mM EDTA, and 0.05% each of bromophenol blue and xylene cyanol), heat-denatured, and electrophoresed on 0.5 × MDE gels ±10% glycerol. Abnormal single stranded DNA detected as autoradiographic shifts were re-amplified by PCR and subjected to automated dye-terminator sequencing (ABI 373).

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Bin1 functionally interacts with Myc and inhibits cell proliferation via multiple mechanisms

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Abstract

The tumor suppressor Bin1 was identified through its interaction with the N-terminal region of Myc which harbors its transcriptional activation domain. Here we show that Bin1 and Myc physically and functionally associate in cells and that Bin1 inhibits cell proliferation through both Myc-dependent and Myc-independent mechanisms. Bin1 specifically inhibited transactivation by Myc as assayed from artificial promoters or from the Myc target genes ornithine decarboxylase (ODC) and α prothymosin (pT). Inhibition of ODC but not pT required the presence of the Myc binding domain (MBD) of Bin1 suggesting two mechanisms of action. Consistent with this possibility, a non-MBD region of Bin1 was sufficient to recruit a repression function to DNA that was unrelated to histone deacetylase. Regions outside the MBD required for growth inhibition were mapped in Ras cotransformation or HepG2 hepatoma cell growth assays. Bin1 required the N-terminal BAR domain to suppress focus formation by Myc whereas the C-terminal U1 and SH3 domains were required to inhibit adenovirus E1A or mutant p53, respectively. All three domains contributed to Bin1 suppression of tumor cell growth but BAR-C was most crucial. These findings supported functional interaction between Myc and Bin1 in cells and indicated that Bin1 could inhibit malignant cell growth through multiple mechanisms.

Introduction

Myc is a central regulator of cell proliferation and apoptosis that is frequently activated in human malignancy (reviewed in Henriksson & Lüscher, 1996; Prendergast, 1997; Facchini & Penn, 1998). In normal cells induced to divide, the levels of Myc increase and remain elevated, indicating it is required throughout the cell cycle for proliferation. Deregulated Myc expression is sufficient to drive quiescent cells into S phase to prevent cell cycle exit. Conversely, suppression of Myc blocks mitogenic signals and facilitates terminal differentiation. Myc can also induce apoptosis, a feature manifested in normal cells when its expression is enforced and uncoupled from the orchestration of other cell cycle regulatory events. Myc is thought to act in the guise of a transcription factor, but the exact mechanisms underlying its oncogenic and apoptotic properties remain obscure.

We previously identified a cellular polypeptide, Bin1, which interacts with the putative transcriptional activation domain of Myc (Sakamuro *et al.*, 1996). The interaction depends upon the integrity of the so-called Myc boxes, two evolutionarily conserved segments which are necessary for both cell transformation and apoptosis. Several observations support the hypothesis that Bin1 is a tumor suppressor that controls cell cycle transit and proliferation. First, Bin1 inhibits cell transformation by Myc or adenovirus E1A (Sakamuro *et al.*, 1996). Second, Bin1 is related to RVS167, a negative regulator of the cell cycle in yeast (Bauer *et al.*, 1993). Third, although widely expressed in normal cells, Bin1 is poorly expressed or undetectable in ~50% of carcinoma cell lines and primary breast carcinomas examined (Sakamuro *et al.*, 1996). Fourth, deficits in expression are functionally significant, because Bin1 can inhibit the growth of tumor cells which lack endogenous expression (Sakamuro *et al.*, 1996). Fifth, similar to several other important tumor suppressors, Bin1 promotes differentiation in the myogenic pathway and its inhibition suppresses differentiation (Wechsler-Reya *et al.*, 1998). Finally, the human Bin1 gene has been mapped to chromosome 2q14 (Negorev *et al.*, 1996), within a mid-2q region that is deleted in ~42% of metastatic prostate cancers (Cher *et al.*, 1996), and recent investigations suggest that loss of Bin1 function may contribute to prostate tumor progression. Evidence from genetic, *in vitro*

biochemical association, and co-localization experiments supports interaction between Bin1 and Myc (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a) but *in vivo* physical association and functional interaction had not been documented (Wechsler-Reya *et al.*, 1997a). In addition, Bin1 was shown to inhibit growth by adenovirus E1A as well as Myc, but whether this reflected similar or different functions was undetermined. In this study, we show that Bin1 physically associates with Myc in cells and inhibits its transcriptional properties and that Bin1 can inhibit malignant cell growth through Myc-independent as well as Myc-dependent mechanisms. These findings support a role for Bin1 in governing the oncogenic properties of Myc but indicate that Bin1 also has additional roles in cell growth regulation.

Results

Physical and functional association of Myc and Bin1 in cells. Coimmunoprecipitation and transcription activation experiments were performed to examine the ability of Myc and Bin1 to functionally associate in cells. Association of Myc and Bin1 was observed by coimmunoprecipitation from baculovirus-infected Sf9 cells and untransfected C2C12 myoblasts, where Bin1 function has been examined (Wechsler-Reya *et al.*, 1998), using NP40 buffer conditions previously shown to support interaction of Myc and Bin1 *in vitro* (150 mM NaCl and 0.1% NP40). Bin1 was extracted more readily than Myc by NP40 lysis buffer from Sf9 cells infected with recombinant c-Myc and Bin1 baculoviruses, consistent with the fact that efficient extraction of Myc requires harsher conditions (RIPA buffer and sonication [Hann *et al.*, 1983]). However, the Myc complexes extracted under these conditions contained Bin1 as indicated by coimmunoprecipitation with Myc antibody (see Figure 1A). Association was specific because co-expression of the negative control proteins RhoB or yeast ADA3 with Bin1 did not result in Bin1 precipitation (data not shown). Bin1 antibodies capable of recognizing native Bin1 protein bind to epitopes in the Myc binding domain (MBD) (Wechsler-Reya *et al.*, 1997a) so the reverse immunoprecipitation experiment was intractable. Experiments using epitope-tagged Bin1 species were inconclusive, because tags at either the C- or N-terminus of Bin1 were not recognized unless denaturing conditions were used (i.e. RIPA buffer) that did not preserve Myc interaction *in vitro* (Sakamuro *et al.*,

1996; data not shown). However, Myc-Bin1 association was similarly observed in C2C12 cells. Myc and Bin1 are each expressed in proliferating C2C12 cells with Bin1 in stochiometric excess (Wechsler-Reya *et al.*, 1998). When C2C12 is induced to differentiate (Blau *et al.*, 1985), Bin1 is upregulated while Myc is downregulated to undetectable levels (Wechsler-Reya *et al.*, 1998), providing a useful negative control for association. As before, Myc was extracted inefficiently by NP40 buffer but Bin1 was detected in Myc complexes that were immunoprecipitated by Myc antibody (see Figure 1B). The presence of Bin1 in these complexes was not due to antibody artifact or another nonspecific cause, because Bin1 was not detected in similar immunoprecipitates prepared from differentiated cell extracts.

To determine whether Bin1 association affected the transcriptional properties of Myc, transient activation assays were performed using a variety of promoters documented to be physiologically activated by c-Myc. The experiments employed luciferase (luc) reporter genes driven by artificial Myc-responsive promoters containing either multimerized DNA binding sites upstream of a minimal viral promoter or by the 5' regions of the Myc target genes ornithine decarboxylase (ODC) and α-prothymosin (pT) (Bello-Fernandez *et al.*, 1993; Eilers *et al.*, 1991). The two artificial reporter genes were p3XMycE1b-luc (Gupta *et al.*, 1993) and Gal_5-E1b-luc, which included either 3 Myc-binding sites or 5 yeast GAL4 binding sites upstream of the adenovirus E1b minimal promoter. The latter reporter was used where activation was driven by chimeric molecules containing Bin1 or the Myc N-terminal transactivation domain (aa 1-262) fused to the DNA binding domain of the yeast transcription factor GAL4 (Kato *et al.*, 1990). The ODC and target gene reporters were ODCΔluc and PrT-luc (Bello-Fernandez *et al.*, 1993; Desbarats *et al.*, 1996; Packham & Cleveland, 1997). Cells were transfected with reporter plasmids and vectors for c-Myc or GAL4-Myc, Bin1, or the MBD deletion mutant Bin1ΔMBD (Sakamuro *et al.*, 1996). Max was included in pT experiments for optimal activation of this gene as documented (Desbarats *et al.*, 1996). Western or Northern analyses confirmed exogenous gene expression in transiently transfected cells (data not shown). ODC activation experiments included as a positive control for N-terminal interaction and inhibition of Myc activation the retinoblastoma (Rb)-related protein p107 (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994).

Bin1 selectively inhibited Myc activation on all the Myc reporter promoters tested (see Figure 2). In NIH3T3 cells, Myc activated p3XMycE1b-luc ~2.5-fold, similar to the level observed by others (Kretzner *et al.*, 1992), and titration of Bin1 into the assay reversed the effect of Myc (see Figure 2A). Similarly, Myc activated the ODC promoter ~2.3-fold, also as documented previously (Packham & Cleveland, 1997), and Bin1 reversed this effect as potently as p107 (see Figure 2B). Deletion of the Myc-binding domain (MBD) from Bin1 relieved its ability to inhibit ODC in both HeLa and NIH3T3 cells (see Figure 2C). The inability of Bin1ΔMBD to suppress Myc was not due to polypeptide instability nor to general loss of function, because Bin1ΔMBD accumulated similarly to wild-type Bin1 in transfected COS cells and because Bin1ΔMBD could inhibit E1A transformation (see below). A more robust activation of pT by Myc/Max was also inhibited by Bin1 ~3-fold (see Figure 2D). Bin1ΔMBD also inhibited Myc activation of pT indicating the effect on this gene was MBD-independent. However, inhibition was specific because Bin1 did not affect activation by VP16 (see below). Western analysis confirmed Myc and Max accumulation in transiently transfected cells, ruling out the trivial possibility that Bin1 acted by inhibiting the exogenous Myc or Max expression (data not shown). The specificity of the effect of Bin1 for the Myc N-terminus was investigated using GAL4-Myc or a second GAL4 chimera which included instead the activation domain from the nonspecific but broadly active herpes virus activator VP16 (GAL4-VP16). For these experiments, we examined activation of a pT reporter (GAL4mE-prT-luc) that was identical to the prT-luc reporter used above except that the two Myc binding sites in the gene were replaced with GAL4 binding sites (Desbarats *et al.*, 1996). Bin1 inhibited activation of pT by GAL-Myc but not by GAL4-VP16 (Figure 2E). Similar results were obtained with GAL4-E1b-luc (data not shown). Taken together, the results of the immunoprecipitation and transcription experiments argued that Bin1 physically and functionally interacted with Myc in cells.

Bin1 can recruit a transcriptional repression function to DNA. Bin1 does not harbor motifs characteristic of transcription adaptor proteins, so one interpretation of the above results was that Bin1 acted via a passive mechanism, for example, by occluding contacts with as yet unidentified coactivators or with the TATA-binding protein (TBP), which has been reported to interact with Myc

(Hateboer *et al.*, 1993). Alternately, Bin1 may act through an active repressive mechanism, perhaps by recruiting a corepressor to the promoter similar to the Mad-binding protein mSin3 (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995). To assess the latter hypothesis, we tested the effects of Bin1 on basal transcription of a promoter to which it was tethered in a Myc-independent manner. This was achieved by fusing Bin1 in frame to the DNA binding domain of GAL4 to generate GAL4-Bin1. A second GAL4 chimera that lacked the MBD was constructed (GAL4-Bin1ΔMBD) to eliminate MBD-dependent interactions with Myc, Myc-binding coactivators yet to be identified, or possibly TBP (Hateboer *et al.*, 1993), all of which might mask repressive effects or make their interpretation more difficult. HeLa cells were transfected with the artificial promoter-reporter gene GAL_5-E1b-luc and equivalent amounts of expression vectors for unfused GAL4 DNA binding domain (GAL0), GAL4-Bin1, or GAL4-Bin1ΔMBD, and cell lysates were processed for luciferase activity as before. GAL4-Bin1 was only slightly inhibitory but GAL4-Bin1ΔMBD repressed basal transcription ~2.5-fold relative to unfused GAL0 (see Figure 3A). GAL4-Bin1ΔMBD had little effect on the activity of luciferase reporters lacking GAL4 sites (data not shown), indicating that this effect was dependent on DNA binding. To determine if repression reflected recruitment of a Bin1-binding factor, we added vector, wild-type (untethered) Bin1, or Bin1ΔMBD to the cotransfection. If the activity was intrinsic, cotransfection of Bin1 would not affect repression, whereas if repression was due to recruitment of a *trans*-acting factor then untethered Bin1 would be predicted to titer the repressive effect. Consistent with the latter case, both Bin1 and Bin1ΔMBD relieved repression by GAL4-Bin1ΔMBD (see Figure 3B). The greater relief provided by Bin1ΔMBD suggested that a region outside of the MBD might recruit a repression function. Experiments in which trichostatin A was added did not relieve the repressive effect of GAL4-Bin1ΔMBD (data not shown), suggesting that this function was not a histone deacetylase and that Bin1 acts differently than mSin3 (Facchini & Penn, 1998). Nevertheless, the results suggested that Bin1 may actively inhibit Myc activation by recruiting a repression function.

Expression and localization of Bin1 deletion mutants. To identify non-MBD regions that are important for Bin1 activity a set of deletion mutants was constructed (see Figure 4). BAR-C and

SH3 encompass regions of Bin1 that are related to the neuron-specific protein amphiphysin and to the yeast cell cycle regulator RVS167 (the BAR nomenclature reflects the Bin1/amphiphysin/RVS167 homology in this region; BAR-C represents the C-terminal half of the BAR domain[see Figure 4A]). The SH3 domain located at the C-terminus is dispensable for interaction with Myc (Sakamuro *et al.*, 1996). The central region is not conserved in amphiphysin or RVS167 and is unique to Bin1. This region includes the so-called unique-1 (U1) region encoded in the human gene by exon 9; the alternately spliced and strongly positively charged unique-3 (U3) region encoded by exon 10; the unique-2 (U2) region encoded by exon 11 which harbors two copies of the SH3 binding motif PXXP; and the MBD (Wechsler-Reya *et al.*, 1997b). The MBD as initially defined encompassed aa 270-389. Deletions of three subsections of this segment were generated for this study, aa 270-288, aa 270-315 (comprising the newly defined U2 region) and aa 323-356 (N-terminal half of the MBD). Expression of the mutant polypeptides was confirmed by immunoprecipitation from COS cell extracts. Cells were transfected with vectors for each mutant, metabolically labeled with 35S-methionine, and extracts were prepared and processed for immunoprecipitation with a mixture of Bin1 monoclonal antibodies (Wechsler-Reya *et al.*, 1997a). The apparent and predicted MWs of the mutants did not coincide in each case because of the presence of a determinant for aberrant gel mobility that maps to the MBD region (Sakamuro *et al.*, 1996). Each mutant was observed to accumulate as efficiently as full-length Bin1 (see Figure 4A). The cell localization of several mutants was examined by indirect immunofluorescence in transiently transfected 293T cells (see Figure 4B). The presence of an SV40 replication origin on the expression vectors made it possible to distinguish cells expressing exogenous proteins by using a higher dilution of Bin1 monoclonal antibody than needed to detect endogenous expression (1:100 instead of 1:5 dilution). Consistent with previous results (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a), wild-type Bin1 localized exclusively to the nucleus, as did Bin1 mutants lacking the U1, U2, U3 and SH3 regions (Bin1ΔU1 was also preferentially excluded from the nucleolus). U3 contains a nuclear localization motif but its dispensability for nuclear localization was consistent with recent findings in which alternate splicing of the exon encoding U3 after myoblast differentiation is correlated with the appearance of cytosolic Bin1 species (Wechsler-Reya *et al.*, 1998; Wechsler-Reya *et al.*, 1997b). Instead, BAR-C contained a critical nuclear localization signal,

because both nuclear and cytosolic staining was detected in cells transfected with Bin1ΔBAR-C. We concluded that BAR-C sequences between aa 125-207 included signal(s) for nuclear localization and/or retention.

Bin1 inhibits malignant cell transformation by multiple mechanisms. Using the Ras cooperation assay performed in primary rat embryo fibroblasts (REFs) (Land *et al.*, 1983; Ruley, 1983), we previously showed that Bin1 inhibits malignant transformation by c-Myc in an MBD-dependent manner (Sakamuro *et al.*, 1996). To define other regions required, REFs were transfected with expression vectors for Myc, oncogenic Ras, and Bin1 or Bin1 deletion mutants, and transformed cell foci were scored two weeks later (see Figure 5). Consistent with previous results (Sakamuro *et al.*, 1996), wild-type Bin1 suppressed focus formation by Myc ~6-fold relative to the empty vector control. Most deletion mutants inhibited focus formation as efficiently as wild-type Bin1, suggesting modularity in the structural organization of this polypeptide. Only BAR-C or the MBD segment aa 323-356 were required, identifying BAR-C determinants as crucial to inhibit Myc transformation along with the MBD. Since aa 270-315 (U2 region) was dispensable for inhibiting Myc transformation the critical part of the MBD therefore was confined to a 66 residue segment between aa 323-389. The inactivity of the MBD aa 323-356 or BAR-C deletion mutants was not due to protein instability, because each polypeptide accumulated similar to wt Bin1 in COS cells (see Figure 4A), nor to misfolding, because each polypeptide efficiently suppressed transformation by E1A or mutant p53 (see below). Bin1ΔBAR-C localized to the nucleus and cytoplasm (see Figure 4B) but its ability to suppress E1A and mutant p53, which act in the nucleus, also argued against mislocalization as the cause for loss of activity against Myc. We previously showed that Bin1 inhibited transformation by adenovirus E1A but not SV40 large T antigen (Sakamuro *et al.*, 1996), and in this study we show that Bin1 also inhibited transformation by dominant inhibitory mutant p53. Bin1 suppressed transformation by E1A or mutant p53 ~3-fold (see Figure 6); the inhibitory effects of each could be titered as was the case with Myc (Sakamuro *et al.*, 1996) by altering the ratio of Bin1 to E1A or mutant p53 in the assay (data not shown). U1 was crucial to inhibit E1A and U1 and SH3 were both crucial to inhibit mutant p53 (see Figure 6). U3, BAR-C, and MBD were each dispensable to inhibit either

oncoprotein. As before, neither protein instability or misfolding was responsible for the loss of activity of either mutant since each accumulated in COS and each could suppress Myc transformation (see Figures 4A and 5). Northern analyses of RNA isolated from pools of foci derived from Myc+Ras, E1A+Ras, or mutant p53+Ras transfections showed that, as predicted, mutant Bin1 messages accumulated in transformed cells if the mutant was biologically inactive. For example, Bin1ΔBAR-C message only accumulated in Myc+Ras foci whereas Bin1ΔU1 message only accumulated in E1A+Ras or mutant p53+Ras foci (data not shown). Thus, the domains required to inhibit E1A and mutant p53 were distinct from those required to block Myc. The importance of the BAR-C domain to the inhibitory activity of Bin1 was confirmed in HepG2 cells (see Figure 7). Deletion of other domains only partly relieved HepG2 growth consistent with the likelihood that multiple growth mechanisms were deregulated in these tumor cells (data not shown). Notably, MBD deletion also only slightly relieved suppression, underscoring the importance of MBD-independent mechanisms for growth inhibition by Bin1. Since neither E1A nor mutant p53 require endogenous Myc to transform cells, the differences in domain dependence argued that Bin1 could regulate malignant cell proliferation through Myc-independent as well as Myc-dependent mechanisms.

Discussion

This study supports the assertion that Myc and Bin1 physically and functionally associate in cells, and it showed that Bin1 can inhibit malignant cell proliferation by both Myc-dependent and Myc-independent mechanisms (see Figure 8). Previous work indicated that Myc-Bin1 complexes were detected by coimmunoprecipitation from recombinant baculovirus-infected Sf9 cells or from naive C2C12 cells. The fact that Myc-Bin1 complexes could be identified in growing C2C12 cells suggested that association is not inhibitory *per se* but may be subjected to posttranslational regulation. This possibility would be consistent with demonstrations that Bin1 is phosphorylated and associated *in vivo* with other proteins in addition to Myc (Wechsler-Reya *et al.*, 1997a). The ability of Bin1 to specifically inhibit Myc function as measured by activation of artificial and natural target genes supported *in vivo* association.

Activation by Myc/Max or by GAL4-Myc chimeras containing the Myc transactivation domain, but not by GAL4-VP16, was susceptible to Bin1 inhibition. VP16 is a complex activator that can act through a variety of adaptors, so the fact that VP16 was not inhibited by Bin1 indicated that its activity was specific and not due to nonselective suppression of transcriptional activation. ODC and pT are two paradigm target genes for Myc and the ability of Bin1 to inhibit each supported the notion of functional interaction. Whether Bin1 has a physiological role in transcription bears further analysis. However, in support of this possibility we showed that Bin1 could recruit a potential repression function to DNA via an MBD-independent interaction. In addition, Myc has been reported to interact *in vitro* with TATA-binding protein (Hateboer *et al.*, 1993) and we have observed that the Bin1 MBD can avidly bind TBP *in vitro* (D.S. and G.C.P., unpublished observations). Although the consequences of Myc-TBP interaction have not been established *in vivo* the ability of Bin1 to bind TBP conceivably represents a second mechanism through which Bin1 could disrupt Myc activation. As considered above, it is possible that the inhibitory effects of Bin1 on Myc activation are passive and an epiphenomenon of steric occlusion of coactivators which are yet to be identified. Recent results indicating that Bin1 is necessary for Myc-mediated apoptosis (D.S., J. Duhadaway, and G.C.P., unpublished observations) would provide a biological foundation to assess the physiological significance of the putative transcriptional properties of Bin1 documented in this study.

The N-terminal BAR-C region of Bin1 was required to inhibit Myc transformation. BAR-C is a charged region of 84 aa predicted to be both α helical and involved in coiled-coil interactions (Lupas, 1996). A key functional role for this region is supported by the fact that it contains the most highly conserved sequences in Bin1 in evolution (G.C.P., unpublished observations). Given the requirement for MBD and BAR-C to suppress Myc transformation one might have expected both regions to be important for the inhibitory effects of Bin1 in HepG2, which overexpresses Myc (G.P., unpublished observations). However, if Myc-independent growth pathways deregulated in HepG2 are dominant or co-dominant with Myc-dependent pathways then this would not be expected to be the case. BAR-C included a signal(s) for nuclear localization or retention, while NLS-like sequences in U3 (Sakamuro *et al.*, 1996) have been shown here and elsewhere (Wechsler-Reya *et al.*, 1998) to be dispensable. The results of this study

mapped the MBD within a 61 residue segment between aa 315-376 immediately upstream of the SH3 domain. Interestingly, this region of Bin1 is encoded by two exons and the more 5' exon has been found to be alternately spliced in cells (Wechsler-Reya *et al.*, 1997b). The aa 323-356 deletion which relieved Myc suppression activity closely overlaps the sequences encoded by this exon. Thus, one alternately spliced Bin1 species in cells probably lacks Myc binding capacity and functions independently of Myc, a likelihood that is consistent with Myc-independent growth inhibitory properties of Bin1 identified in this study.

The C-terminal U1 and SH3 regions were required to inhibit transformation by E1A or p53 but not by Myc. U1 is contained on a single exon which encodes 28 aa (Wechsler-Reya *et al.*, 1997b). E1A transforms cells by displacing E2F from Rb (Dyson & Harlow, 1992) so U1 either impedes this process somehow or acts downstream to interfere with E2F effectors. Consistent with a link between U1 and the Rb/E2F system, U1 deletion also blocks transformation by the human papilloma virus E7 protein (data not shown), which acts similarly to E1A by interfering with Rb/E2F interaction (Phelps *et al.*, 1988). The requirement of U1 to inhibit mutant p53 is consistent with evidence that cell transformation by mutant p53 also depends on interference with Rb/E2F interactions (Hansen *et al.*, 1995). The SH3 domain of Bin1 was also necessary to inhibit transformation by mutant p53. To our knowledge Bin1 and Abl are the only two SH3-containing proteins localized to the nucleus, and recently Abl has been shown to interact with Bin1 in an SH3-dependent manner((Kadlec & Pendergast, 1997); D.S. and G.C.P., unpublished observations). This may be of consequence since Abl and p53 have been reported to interact in cells (Yuan *et al.*, 1996), although the physiological significance of this interaction has not been established clearly. Direct interaction between the Bin1 SH3 and the PxxP motifs in the apoptosis effector domain of p53 (Sakamuro *et al.*, 1997) could be germane since PxxP motifs constitute SH3 ligands. Indeed, since this region also has been implicated in growth inhibition (Walker & Levine, 1996), and the transforming efficiency of mutant p53 rests upon more than simple inactivation of endogenous p53 (Dittmer *et al.*, 1993; Hulboy & Lozano, 1994), it is conceivable that mutant p53 may promote transformation in a PxxP-dependent manner by sequestering a nuclear SH3-containing growth suppressors such Bin1.

Materials and Methods

Plasmid constructions. The following plasmids have been described. CMV-Bin1 and CMV-Bin1ΔMBD encode full-length Bin1 or an MBD deletion mutant, respectively (Sakamuro *et al.*, 1996). LTR Hm contains a Moloney retroviral long terminal repeat-driven normal human c-*myc* gene (Kelekar & Cole, 1986); pSVLneo-C-myc is an SV40 early region-driven c-Myc vector used in Figure 2A that has been described (Zhang & Prochownik, 1997); p1A/neo contains the 5' end of the adenovirus type 5 genome including the E1A region (Maruyama *et al.*, 1987); LTR p53ts encodes a temperature-sensitive dominant inhibitory mutant of murine p53 (Michalovitz *et al.*, 1990); and pT22 contains an activated H-ras gene (Land *et al.*, 1983). CMV-p107 contains a full-length human p107 cDNA (Zhu *et al.*, 1993) in the cytomegalovirus enhancer/promoter-containing vector pcDNA3 (Invitrogen). p3XMyc-E1b-luc is an artificial reporter gene containing multimerized CACGTG Myc E box sequences upstream of the minimal adenovirus E1b promoter (Gupta *et al.*, 1993). GAL4-E1b-luc and GAL4-SV40-luc are GAL4 reporters which contain multimerized GAL4 sites upstream of the minimal E1b or SV40 early promoters (gifts of F. Rauscher III). The ODC luciferase reporter ODCΔluc and the α-prothymosin luciferase reporters PrT-luc and GAL4mE-PrT-luc have been described (Desbarats *et al.*, 1996; Packham & Cleveland, 1997). The BacBin baculovirus was prepared by standard methods (O'Reilly *et al.*, 1992) using the baculovirus expression vector pVL1392 (Invitrogen) into which the full-length Bin1 cDNA was subcloned. A murine c-Myc baculovirus (a gift of M. Cole) was prepared similarly. GAL0 is the DNA binding domain of GAL4 (aa 1-143) and GAL4-Myc includes aa 1-262 of human Myc except the b/HLH/LZ region (Kato *et al.*, 1990) which is necessary to bind Max (Prendergast *et al.*, 1991). Bin1 deletion mutants and GAL4 fusion genes were subcloned for expression in pcDNA3 (the same vector as Bin1 and Bin1ΔMBD). Bin1ΔBAR-C was constructed by dropping an internal Afl III restriction fragment from CMV-Bin1, resulting in a deletion of aa 125-207 from the BAR domain (Sakamuro *et al.*, 1996). The remaining mutants were generated by standard PCR methodology using the oligonucleotide primers 995'(Bam), 993'SH3(Xho) (Sakamuro *et al.*, 1996) and others whose sequence is derived from the Bin1 cDNA sequence (GenBank accession number U68485). The integrity of PCR-generated fragments was verified

by DNA sequencing. To conserve space, oligonucleotides and construction details are omitted but are available from G.C.P. Bin1ΔU1 lacks aa 224-248; Bin1ΔNLS, aa 251-269; Bin1ΔSH3, aa 384-451; the other mutants lack the residues indicated. GAL4-Bin1 fusions were generated in two steps by first subcloning the 143 aa DNA binding domain from GAL0 into pcDNA3 and then ligating in-frame full-length Bin1 or Bin1ΔMBD (lacking aa 270-383) cDNAs downstream.

Cell culture. COS, 293T, HeLa, and HepG2 cells from the ATCC were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (Atlantic) and 50 U/ml penicillin and streptomycin (Fisher). NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum (Gibco) and antibiotics (transfections were performed in media containing 10% fetal calf serum). C2C12 cells were carried in DMEM supplemented with 15% fetal bovine serum and antibiotics. Differentiation of C2C12 was induced by removing shifting cells at ~70% confluence to DMEM supplemented with 5% horse serum and antibiotics for 5 days, when myotube formation was maximal. Secondary passage rat embryo fibroblasts (REFs) were obtained from Whittaker Bioproducts and cultured and transfected as described (Prendergast *et al.*, 1992). For transformation assays, secondary passage REFs seeded in 10 cm dishes were transfected overnight by a calcium phosphate coprecipitation method (Chen & Okayama, 1987) with 5 μg each of oncogenic Ras plus Myc, E1A, or mutant p53 expression plasmids and 10 μg of Bin1 plasmid or empty vector. Cells were fed and the next day passaged into 1 15 cm dish (Myc transfections) or 3 10 cm dishes (E1A or mutant p53 transfections). Foci were scored by methanol fixation and crystal violet staining 12-16 days later. Colony formation assays in HepG2 cells were performed by seeding ~3 x 10^5 cells in 6 cm dishes and transfecting the next day with 2 μg plasmid DNA using Lipofectamine (Gibco/BRL). Cells were passaged 48 hr after transfection at a 1:10 ratio into 6 cm dishes containing media with ~0.6 mg/ml G418 and cell colonies were scored by crystal violet staining ~3 weeks later.

Immunoprecipitation. For insect cell experiments, ~10^7 Sf9 cells were infected with the recombinant baculoviruses indicated at an m.o.i. of approximately 10. Two days after infection, cells

were harvested and ~2 x 10^6 cells for each IP were extracted in 0.5 ml 50 mM TrisCl pH 8/150 mM NaCl/0.1% NP40. Clarified lysates were subjected to immunoprecipitation by incubation 1.5 hr at 4°C with 1 μg of anti-murine c-Myc antibody #6-213 (Upstate Biotechnology) or 100 μl hybridoma supernatant containing the Bin1 monoclonal antibody 99D (Wechsler-Reya *et al.*, 1997a). Immune complexes were collected on Protein G-Sepharose (Pharmacia), washed 4 times with binding buffer, eluted by boiling in SDS gel loading buffer, and fractionated by SDS-PAGE. Gels were Western blotted by standard methods (Harlow & Lane, 1988) and probed with 1 μg/ml anti-Myc or a 1:50 dilution of 99D hybridoma supernatant. Blots were developed using a chemiluminescence kit (Pierce). For experiments in mouse cells, 5-10 dishes of growing or differentiated C2C12 cells were trypsinized, washed with excess growth media and then with 30 ml PBS each at 4°C. All subsequent steps were performed on ice or at 4°C. Cells were resuspended in hypotonic buffer (10 mM HEPES pH 8.0, 10 mM KCl, 0.1 mM EDTA, and 1 mM PMSF, aprotinin, leupeptin, antipain), incubated 3-5 min, and pelleted. These swollen cells were resuspended in extraction buffer (20 mM HEPES, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 0.1% NP-40, and protease inhibitors) and lysed by 10 strokes with a B pestle homogenizer. Before immunoprecipitation, the extract was incubated 15 min and clarified by a 5 min microcentrifugation. The protein concentration in the extract was determined by Bradford assay and 1.5 mg was incubated overnight with 1 μg anti-c-myc #sc-42 (Santa Cruz Biotechnology). Immune complexes were collected on protein G-agarose, washed three times with extraction buffer, and fractionated by nonreducing SDS-PAGE. Gels were Western blotted and probed as indicated with a 1:50 dilution of 99D hybridoma supernatant or ~1 μg/ml anti-Myc antibody 9E10 (Evan *et al.*, 1985). Blots were developed in these experiments with a secondary goat anti-mouse antibody conjugated to alkaline phosphatase, using an colormetric staining reaction catalyzed by this enzyme. To confirm expression of Bin1 deletion mutants, COS cells were metabolically labeled for 2 hr in DMEM lacking methionine and cysteine (Gibco) with 100 μCi/ml EXPRESS labeling reagent (NEN) and cell extracts were prepared with NP40 buffer containing leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and antipain (Harlow & Lane, 1988). Extracts were microcentrifuged for 15 min at 4°C before use. Extracts were precleared by a 1 hr treatment with prebleed sera or normal mouse IgG and 20 μl of a 1:1 slurry of protein G-Sepharose beads at 4°C on a nutator

(Pharmacia). A mixture of hybridoma supernatants (50 µl each) containing Bin1 monoclonal antibodies 99D, 99E, and 99I were used for immunoprecipitation (Wechsler-Reya *et al.*, 1997a). After incubation 1 hr at 4°C, immune complexes were collected on protein G-Sepharose, washed four times with NP40 buffer, eluted in SDS gel loading buffer, fractionated on 10% SDS-PA gels, and fluorographed.

Immunofluorescence. 293T were seeded onto glass cover slips in 6 well dishes and transfected the next day with 5 µg of the Bin1 expression vectors indicated. Two days later, cells were fixed, lysed, and processed for Bin1 immunofluorescence as described previously (Prendergast & Ziff, 1991; Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a), except that a 1:100 instead of a 1:5 dilution of 99D was used to limit detection to cells overexpressing the gene products of interest. Cells were photographed on a Leica immunofluorescence microscope apparatus using Ektachrome film and slides were scanned and processed with Photoshop software.

Transactivation assays. Conditions for transient Myc activation assays were taken from the reports using the various reporter genes employed (Bello-Fernandez *et al.*, 1993; Desbarats *et al.*, 1996; Kato *et al.*, 1990; Packham & Cleveland, 1997; Zhang & Prochownik, 1997). NIH3T3 or HeLa cells were transfected using standard calcium phosphate methods and promoter sequences and total plasmid DNA in each transfection was equalized with empty vectors as appropriate. Each DNA mixture included equivalent amounts of a ß-galactosidase vector to normalize for transfection efficiency. Two days after transfection, cell extracts were prepared and analyzed for luciferase and ß-galactosidase activity using commercial kits, following protocols provided by the vendor (Promega).

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Figure Legends

Figure 1. Biochemical association of Bin1 and Myc in cells. (A.) Association in Sf9 cells. Extracts from $2x10^6$ cells infected with the recombinant baculoviruses indicated were prepared and subjected to IP/Western analysis as described in the text and the Materials and Methods. Dots indicate the position of coprecipitating antibodies recognized by anti-mouse or anti-rabbit secondary antibodies used to develop the blots, by a chemiluminescence technique. (B.) Association in naive C2C12 myoblasts. Extracts from growing (d0) or differentiated (d5) C2C12 cells were prepared and subjected to Western or IP/Western analyses as described in the text and the Materials and Methods. The left panel is a Western blot of an SDS gel loaded with 50 μg extract from d0 or d5 cells, demonstrating constitutive Bin1 expression and the appearance of a larger alternately spliced species in differentiated cells (Wechsler-Reya *et al.*, 1998). The dot indicates a nonspecific band. The middle panel is a Western blot of a nonreducing SDS gel loaded with a Myc immunoprecipitate (sc-42) generated from 1.5 mg of d0 or d5 extracts and probed with a second anti-Myc antibody (9E10). The right panel is a Western blot of a nonreducing SDS gel loaded with 50 μg of d0 extract alone or a Myc immunoprecipitate (sc-42) from 1.5 mg d0 or d5 extracts and probed with anti-Bin1 99D.

Figure 2. Bin1 specifically inhibits gene activation by Myc. (A.) Inhibition of a Myc-responsive artificial promoter. NIH3T3 cells were transfected with the plasmids indicated by standard calcium phosphate method and processed for normalized luciferase activity as described (Zhang & Prochownik, 1997). The data represent the results of three trials each performed in duplicate. (B.) Inhibition of ODC activation. NIH3T3 cells seeded into 6 well dishes were transfected with 1.5 μg of the ODC reporter ODCΔluc, 3 μg of the human c-Myc vector LTR Hm, 1.5 μg CMV Bin1 or CMV p107, and 0.5 μg CMV-ßgal (to normalize for transfection efficiency). pcDNA3 was added to equalize the amount of plasmid in each transfection. Two days later cell extracts were prepared and processed for normalized reporter activity. The graph depicts relative

luciferase activity based on reporter only (set at 100%); the absolute values ranged from 10^3-10^4 light units. The results represent the average of two trials each performed in duplicate. (C.) MBD is required for ODC inhibition. NIH3T3 or HeLa cells were transfected with 0.5 μg ODCΔluc and 2 μg LTR-Hm plus 3.25 μg vector, CMV-Bin1, or CMV-Bin1ΔMBD. Cell extracts were prepared and processed as above. The results represent the average of two trials performed in duplicate. Relative luciferase activity is depicted as the proportion of reporter plus LTR Hm; the absolute values ranged from 10^3-10^4 light units. (D.) Inhibition of pT activation. HeLa cells were transfected with PrT-luc, a ß-galactosidase normalization plasmid, and the vectors indicated as described (Desbarats *et al.*, 1996). Where indicated Bin1 or control plasmids were included in a 1:1 w/w ratio with Myc. Relative luciferase activity is depicted as above; the absolute values ranged from 10^4-10^6 light units. (E.) Bin1 inhibits GAL4-Myc but not GAL-VP16. HeLa cells were transfected with GAL4mE-PrT-luc and the genes indicated as above and processed for relative luciferase activity.

Figure 3. Bin1 recruits a repression function when tethered to a promoter. (A.) Intrinsic repressive quality of Bin1. HeLa cells were transfected with 2 μg GAL4-SV40-luc reporter and 4 μg of the indicated GAL4 chimeric gene and normalized luciferase activity was determined two days later. The data represent the results of at least four trials each performed in duplicate. (B.) The repressive activity of BIN can be titered. Cells were transfected with 2 μg GAL4-SV40-luc reporter, 4 μg of GAL4-Bin1ΔMBD, 4 μg CMV vector, Bin1, or Bin1ΔMBD plasmids and normalized luciferase activity was determined two days later. The data represent the results of four trials each performed in duplicate.

Figure 4. Structure, expression, and localization of Bin1 deletion mutants. (A.) Expression of Bin1 mutants. COS cells were transfected with the expression vectors indicated, metabolically labeled with ^{35}S-methionine/cysteine, and cell extracts were prepared and subjected to immunoprecipitation with Bin1 monoclonal antibodies. Immunoprecipitates were examined by

SDS-PAGE and fluorography. The bars at the bottom of the cartoon denote regions that are structurally related among proteins of the BAR family, which includes Bin1, amphiphysin, and RVS167 (Sakamuro *et al.*, 1996). (B.) Localization of Bin1 mutants. 293T cells seeded on glass cover slips were transiently transfected with the expression vectors and processed for indirect immunofluorescence with Bin1 monoclonal antibody 99D as described in the Materials and Methods.

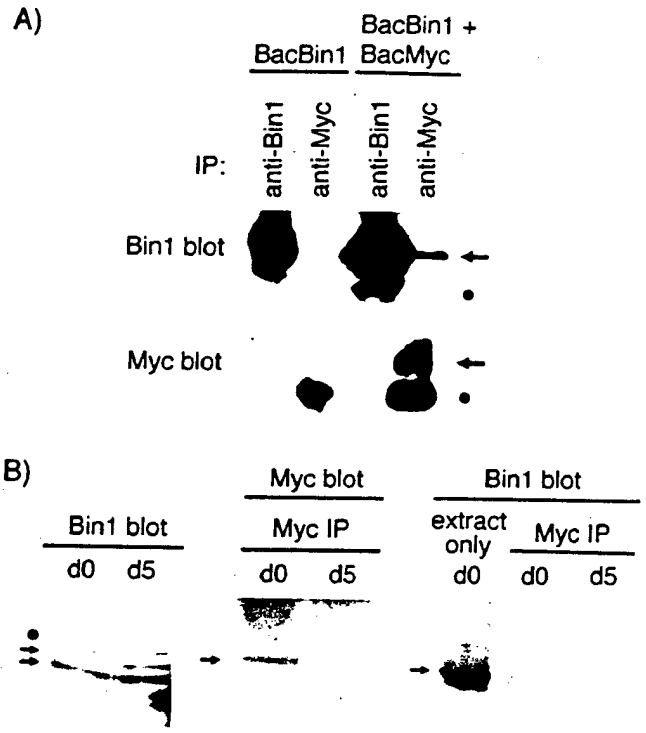
Figure 5. BAR-C is required to inhibit Myc transformation. REFs were transfected with 5 μg each oncogenic Ras and deregulated human c-Myc plasmids plus 10 μg each of the vectors indicated. Transformed cell foci were scored 12-14 days later. The data depict the percentage of Myc+Ras foci formed in the presence of empty vector.

Figure 6. U1 is required to inhibit E1A transformation and U1 and SH3 are each required to suppress mutant p53 transformation. REFs were transfected with 5 μg each oncogenic Ras and adenovirus E1A or dominant inhibitory p53 mutant plasmids plus 10 μg each of the vectors indicated. Transformed cell foci were scored 12-16 days later. The data depict the percentage of E1A+Ras or mutant p53+Ras foci formed in the presence of empty vector.

Figure 7. BAR-C is crucial to suppress tumor cell growth. HepG2 cells were transfected with 2 μg of neomycin (neo^r)-resistance gene marked vectors. G418-resistant cell colonies were scored ~3 weeks later by methanol fixation and crystal violet staining. A representative assay is shown from multiple experiments performed in triplicate.

Figure 8. Summary of Bin1 functions. Myc interaction data is from Sakamuro *et al.*, 1996.

Fig. 1


A)
BacBin1
BacBin1 + BacMyc
IP:
anti-Bin1
anti-Myc
anti-Bin1
anti-Myc
Bin1 blot
Myc blot
B)
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d5
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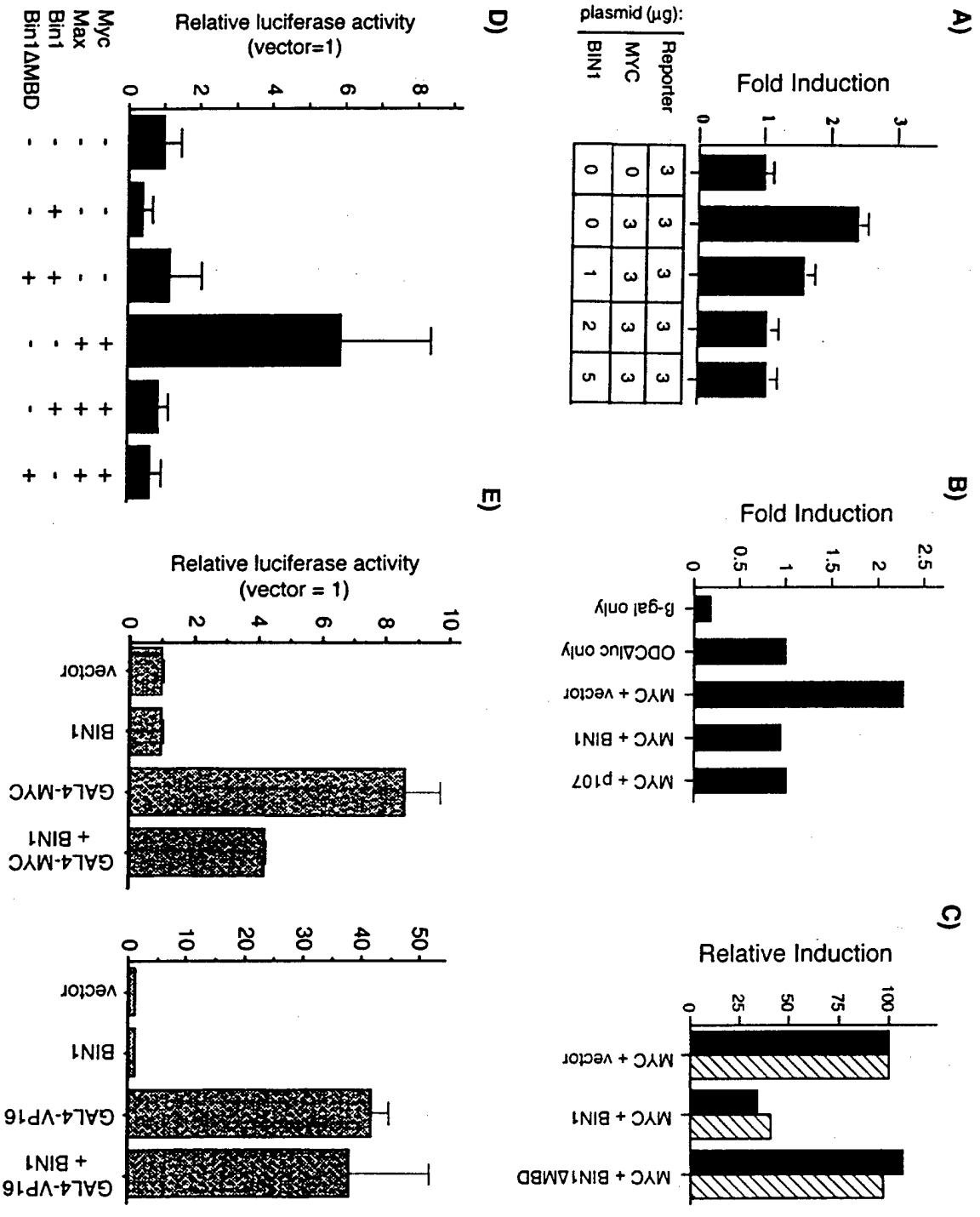
d0
d5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plasmid (µg):
BIN1
MYC
Reporter
Fold Induction
B)
Fold Induction
β-gal only
ODCΔluc only
MYC + vector
MYC + BIN1
MYC + p107
C)
Relative Induction
MYC + vector
MYC + BIN1
MYC + BIN1ΔMBD
D)
Relative luciferase activity
(vector=1)
Myc
Max
Bin1
Bin1ΔMBD
E)
Relative luciferase activity
(vector = 1)
vector
BIN1
GAL4-MYC
GAL4-MYC + BIN1
vector
BIN1
GAL4-VP16
GAL4-VP16 + BIN1

Fig. 3

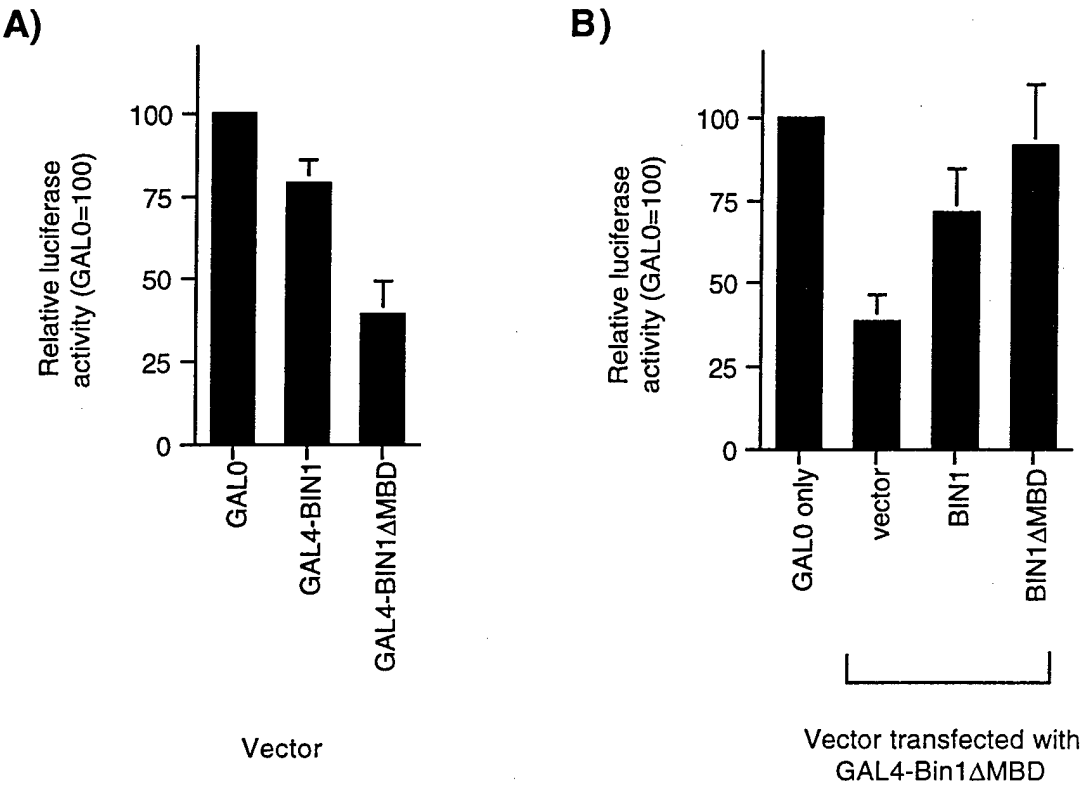

A)
Relative luciferase activity (GAL0=100)
100
75
50
25
0
GAL0
GAL4-BIN1
GAL4-BIN1ΔMBD
Vector
B)
Relative luciferase activity (GAL0=100)
100
75
50
25
0
GAL0 only
vector
BIN1
BIN1ΔMBD
Vector transfected with GAL4-Bin1ΔMBD

Fig. 4A

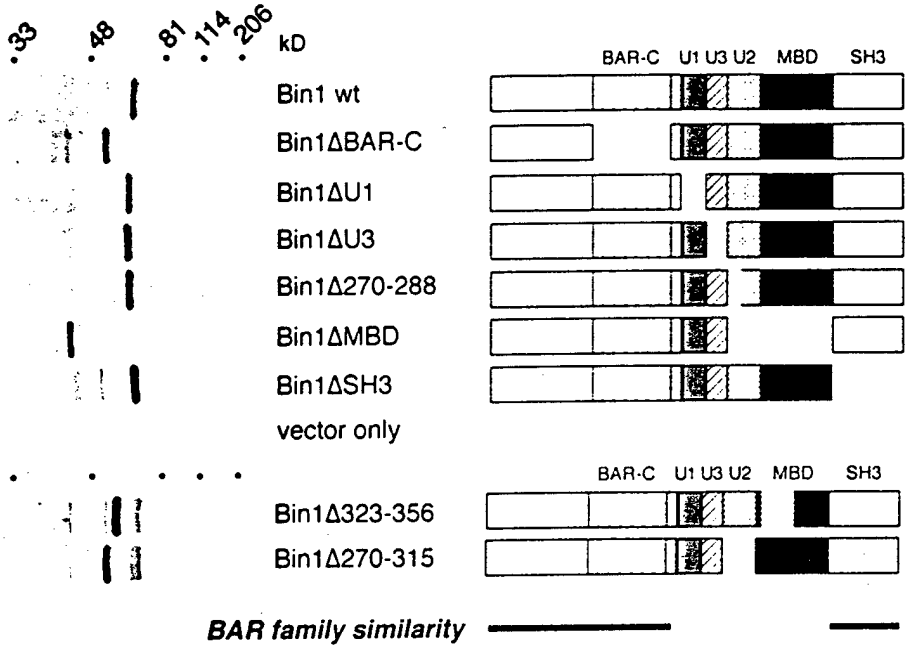
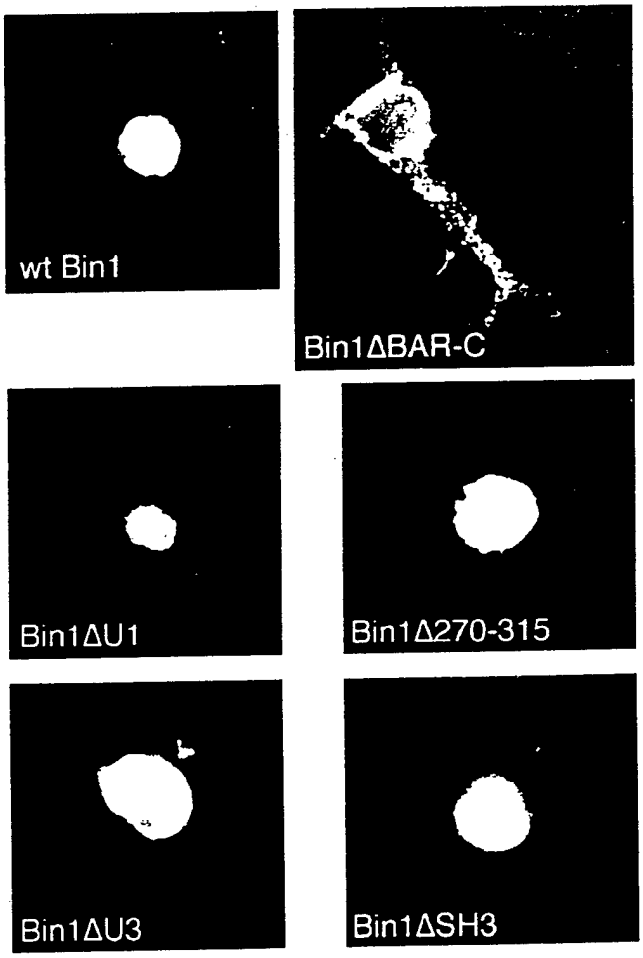

33
48
81
114
206
kD
BAR-C U1 U3 U2 MBD SH3
Bin1 wt
Bin1ΔBAR-C
Bin1ΔU1
Bin1ΔU3
Bin1Δ270-288
Bin1ΔMBD
Bin1ΔSH3
vector only
BAR-C U1 U3 U2 MBD SH3
Bin1Δ323-356
Bin1Δ270-315
BAR family similarity

Fig. 4B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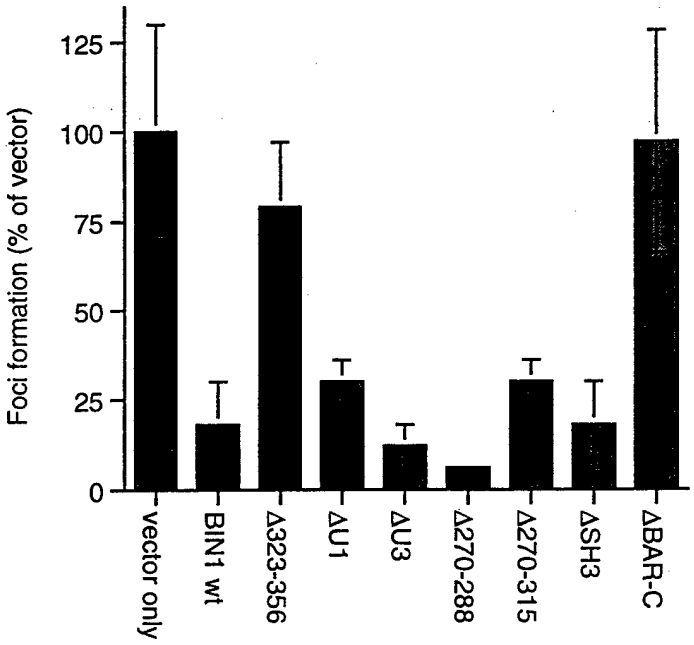
Bin1ΔBAR-C
Bin1ΔU1
Bin1Δ270-315
Bin1ΔU3
Bin1ΔSH3

Fig. 5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100
75
50
25
0
vector only
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Δ323-356
ΔU1
ΔU3
Δ270-288
Δ270-315
ΔSH3
ΔBAR-C

Fig. 6

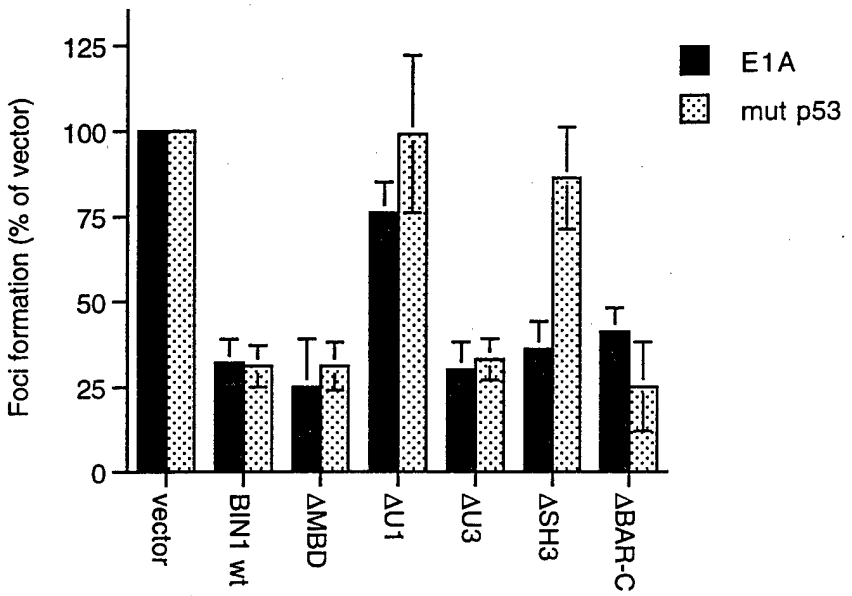

Foci formation (% of vector)
125
100
75
50
25
0
E1A
mut p53
vector
BIN1 wt
ΔMBD
ΔU1
ΔU3
ΔSH3
ΔBAR-C

Fig. 7

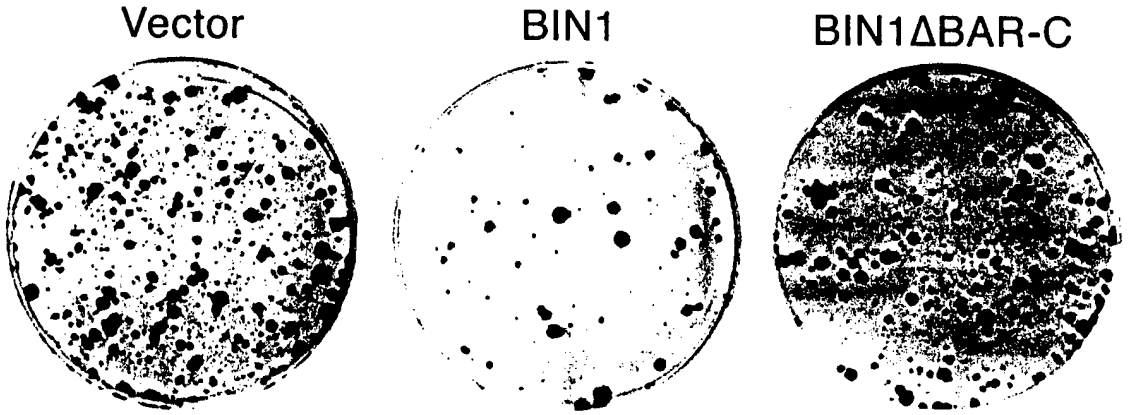
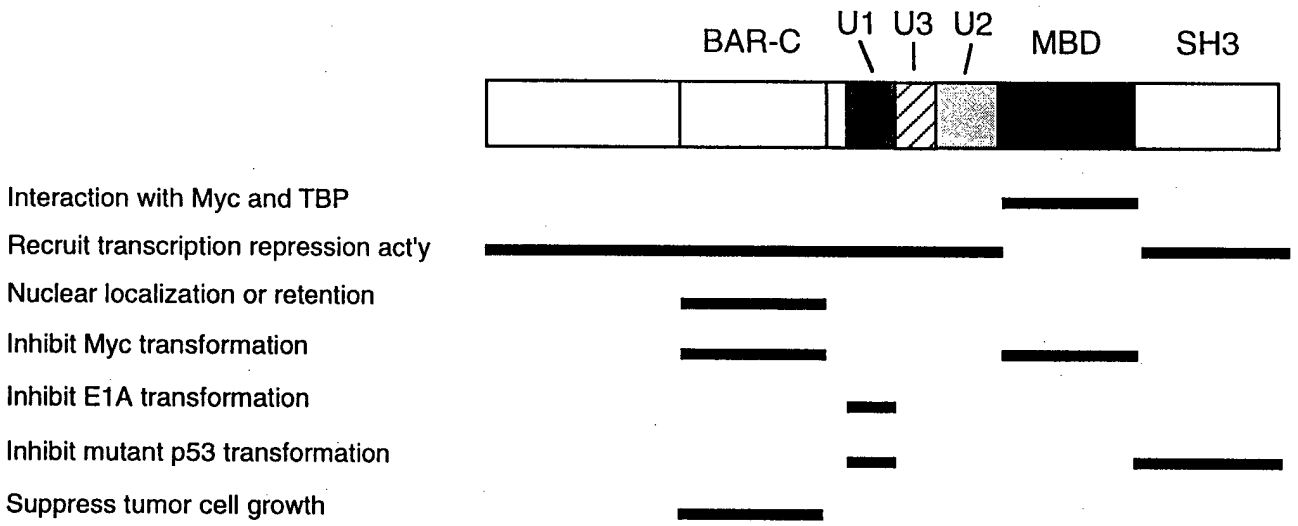


Fig. 8


BAR-C
U1
U3
U2
MBD
SH3
Interaction with Myc and TBP
Recruit transcription repression act'y
Nuclear localization or retention
Inhibit Myc transformation
Inhibit E1A transformation
Inhibit mutant p53 transformation
Suppress tumor cell growth

c-Myc mediates apoptosis through the adaptor protein Bin1

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Abstract

The c-Myc oncoprotein induces proliferation, malignant transformation, and apoptosis but how it drives these diverse processes is unclear. In this study, we report that the c-Myc-interacting adaptor protein Bin1 has a specific and necessary role in the mechanism by which c-Myc induces apoptosis. Expression of antisense or dominant inhibitory Bin1 genes in primary chick fibroblasts had no effect on c-Myc-dependent proliferation or malignant transformation but significantly reduced the susceptibility of cells to c-Myc-induced apoptosis. In particular, overexpression of the c-Myc-binding domain of Bin1 rendered cells resistant to apoptosis, implying that c-Myc-Bin1 interaction is specifically required for death and that Bin1 is part of a death effector mechanism. In baby rat kidney epithelial cells transformed by deregulated c-Myc and mutant p53 (BRK myc/p53ts cells), Bin1 inhibition promoted proliferation and blocked induction of p53-independent cell apoptosis caused by serum deprival. Colony formation assays showed that Bin1 inhibition masked the cytotoxic effects of c-Myc as potently as Bcl-2, supporting productive cell proliferation under low serum conditions. We concluded that Bin1 mediated a death or death sensitization signal from c-Myc. Our findings support the 'dual signal' model for Myc function by distinguishing its transforming and proapoptotic activities on the basis of interactions with a binding protein. We propose that loss of Bin1 in cancer cells may contribute to c-Myc deregulation by abolishing a mechanism that limits its ability to drive cell proliferation inappropriately.

Introduction

c-Myc is a nuclear oncoprotein that is necessary and sufficient to promote efficient cell cycle progression (reviewed in Henriksson and Lüscher 1996; Prendergast 1997; Facchini and Penn 1998). When deregulated by chromosomal translocation, point mutation, gene amplification, or overexpression, c-Myc contributes strongly to the malignant development of many human tumors (Cole 1986). Interestingly, under certain conditions deregulated c-Myc can also induce programmed cell death, or apoptosis (Askew *et al.* 1991; Evan *et al.* 1992). For example, following growth factor deprivation, cells that contain normal c-Myc downregulate its expression and exit the cell cycle, whereas cells that contain deregulated c-Myc maintain its expression and undergo apoptosis. The molecular mechanism(s) underlying the transforming and apoptotic properties of c-Myc have not been elucidated.

Two general models for apoptosis by c-Myc have been considered, termed the conflict and dual signal models (reviewed in Evan *et al.* 1995; Packham and Cleveland 1995). In the conflict model, apoptosis is an indirect response of the cell to an inappropriate growth signal from c-Myc. In the dual signal model, c-Myc is proposed to directly regulate growth and death pathways by interacting with effector functions specific for each process. A variant of the dual signal model proposes that c-Myc does not signal death but instead sensitizes cells to death by other agents (Evan and Littlewood 1998). In either case, the dual signal model would be favored if the proliferative and apoptotic properties of c-Myc were separable. Max interaction is necessary for both processes (Amati *et al.* 1993; Amati *et al.* 1993) but specific roles for other c-Myc-interacting proteins have not been explored.

Bin1 (Box-dependent myc-INteracting protein-1 or Bridging INtegrator-1) is a nucleocytoplasmic adaptor protein that was identified initially through its ability to interact with c-Myc (Sakamuro *et al.* 1996). While its roles as an adaptor are complex, there is significant evidence

supporting a role for Bin1 in cell growth control. Bin1 functionally associates with c-Myc in cells and selectively inhibits its oncogenic and transactivation properties (Elliott *et al.* 1999; Sakamuro *et al.* 1996). In addition, Bin1 inhibits the growth of many human tumor cell lines (DuHadaway *et al.* 1999; Elliott *et al.* 1999; Elliott *et al.* 1999; Ge *et al.* 1999; Ge *et al.* 1999; Sakamuro *et al.* 1996) and similar to other tumor suppressors has been shown to be necessary for myoblast differentiation (Mao *et al.* 1999; Wechsler-Reya *et al.* 1998). Expression of the Bin1 gene (Wechsler-Reya *et al.* 1997) is missing or epigenetically altered in cancer, such as breast and prostate tumors and melanoma (DuHadaway *et al.* 1999; Elliott *et al.* 1999; Ge *et al.* 1999; Ge *et al.* 1999; Wechsler-Reya *et al.* 1997). In this study, we show that c-Myc requires Bin1 to induce apoptosis and that interaction with Bin1 is implicated in mediating a p53-independent death or death sensitization signal from c-Myc. Our results support the 'dual signal' model for Myc function by showing that the transforming and apoptotic properties of c-Myc can be separated on the basis of interactions with a binding protein. We propose that Bin1 contributes to an abortive mechanism which limits the consequences of Myc deregulation in cells, one which is ablated or suppressed in neoplastic settings where Myc is deregulated.

Results

Bin1 interaction is required to mediate apoptosis by c-Myc

Previous biochemical and genetic experiments demonstrated that the c-Myc-binding domain (MBD) of Bin1 was necessary and sufficient for interaction c-Myc and that it was able to dominantly interfere with c-Myc-Bin1 interaction when overexpressed (Sakamuro *et al.* 1996). Therefore, we overexpressed the MBD as a strategy to interfere with Bin1 activity and determine whether the c-Myc-Bin1 interaction was required for c-Myc to induce apoptosis. Primary chick embryo fibroblasts (CEFs) were selected as a model system for several reasons. c-Myc deregulation is sufficient to induce both transformation and apoptosis in this model so one can examine the effects of Bin1

inhibition on each. CEFs undergo c-Myc-induced apoptosis the same way as rodent fibroblasts, displaying characteristic cell detachment, blebbing, chromatin condensation, and DNA degradation (Crouch *et al.* 1996). A major advantage of this system is the availability of replication-competent retroviral vectors (Petropoulos and Hughes 1991) which permit rapid and unselected gene transfer to large populations of cells, thereby greatly reducing selection for antiapoptotic background (a situation which develops in Myc-expressing cells after several weeks of culture). Bin1 is ubiquitously expressed in cells (Sakamuro *et al.* 1996; Wechsler-Reya *et al.* 1997). Expression of Bin1 in CEFs was confirmed with anti-Bin1 monoclonal antibody 99D (Wechsler-Reya *et al.* 1997) by Western analysis and nuclear localization was confirmed by indirect immunofluorescence (data not shown). In the initial experiments, CEFs were infected with combinations of A or B envelope subtype viruses, in which the A subtype viruses carried human c-Myc cDNA or no insert, and the B subtype viruses carried a Bin1 MBD cDNA, a human Bcl-2 cDNA (as a positive control for suppression of apoptosis by c-Myc (Bissonnette *et al.* 1992; Fanidi *et al.* 1992; Wagner *et al.* 1993; Wang *et al.* 1993), or no insert. Three days after infection, cells were harvested to verify expression of the transgenes or to test in growth and apoptosis assays.

Overexpression of the Bin1 MBD did not block the ability of c-Myc to drive CEF proliferation or to induce anchorage-independence, but rendered CEFs resistant to apoptosis by c-Myc following serum deprival almost as well as Bcl-2. Expression of transgenes in CEFs infected with A and B retroviral vectors was confirmed by Northern (data not shown) and Western analysis (see Figure 1A). Growth curves confirmed that deregulation of c-Myc promoted CEF proliferation as expected (see Figure 1B). Bcl-2 coexpression retarded the growth of c-Myc-expressing cells slightly consistent with its cell growth and cell cycle inhibitory effects (O'Reilly *et al.* 1996; Pietenpol *et al.* 1994). In contrast, MBD coexpression-expression slightly promoted proliferation in the presence of deregulated c-Myc. This effect was subtle but reproducible in different trials. To assess the effects of MBD expression on c-Myc-dependent transformation, cells were seeded into soft agar culture to assay anchorage-independent growth. All cells which expressed deregulated c-

Myc formed colonies whereas cells lacking deregulated c-Myc did not exhibit this ability (see Figure 1C). Thus, MBD did not inhibit the growth- and transformation-promoting properties of c-Myc in CEFs. To test the effects of MBD expression on apoptosis by c-Myc, we cultured cells in growth media or in low-serum media which elicits apoptosis in the presence of deregulated c-Myc. Similar to primary rodent embryo fibroblasts (Evan *et al.* 1992), and as shown by others (Crouch *et al.* 1996), CEFs overexpressing c-Myc exhibited massive signs of apoptosis within 24 hr of serum withdrawal (see Figure 2A). Strikingly, coexpression-expression of MBD suppressed apoptosis by c-Myc almost as potently as Bcl-2. The inhibitory effect was specific, because MBD did not inhibit the basal level apoptosis of cells which lacked c-Myc overexpression when they were deprived of serum. Moreover, MBD had little effect on apoptosis induced by thapsigarin (see Figure 2B), which kills through a Ca^{+2}-dependent and c-Myc-independent mechanism that is susceptible to suppression by Bcl-2 (Distelhorst and McCormick 1996). Experiments using c-Myc vectors with promoters of different strength (i.e. RCAS versus RCOS vectors (Petropoulos and Hughes 1991)) demonstrated that the inhibitory effects of MBD could be titered as c-Myc expression was driven to higher levels (data not shown). These results were consistent with the expectation that MBD acted by competing with endogenous Bin1 for interactions with c-Myc. We concluded that Bin1 interaction with c-Myc was necessary for apoptosis but not for transformation.

To corroborate these observations a set of similar experiments was performed using an antisense gene (Bin1 AS) or an inactive effector mutant that dominantly inhibited Bin1 through a different mechanism of action (Bin1Δ4). The inhibitory activity of the Bin1 AS gene against Bin1 has been documented previously in mouse cells (Wechsler-Reya *et al.* 1998). Bin1Δ4 is a deletion mutant that lacks 5 residues in the N-terminal BAR-C domain which is crucial along with the MBD for Bin1 to inhibit c-Myc/Ras cotransformation of rat embryo fibroblasts (Elliott *et al.* 1999). The Δ4 region maps to the most highly conserved part of the Bin1 gene (G.C.P., unpublished observations) and its deletion renders Bin1 inactive but capable of dominant interference with wild-type Bin1 in c-Myc/Ras cotransformation assays (see Figure 3A). Bin1Δ4 has an intact MBD and its

dominant inhibitory activity is based on interactions with proteins other than c-Myc. CEFs expressing c-Myc and Bin1 AS, Bin1Δ4, or no insert were generated by infection with recombinant retroviruses. Exogenous expression was confirmed by RT-PCR to distinguish the Δ4 transcript and (see Figure 3B) and by Northern and Western analysis (data not shown). Similar to what was seen with MBD overexpression, Bin1 AS or Bin1Δ4 did not affect the ability of c-Myc to drive CEF proliferation or transformation, as measured by growth curve determination and acquisition of anchorage-independence (see Figures 3C and 3D). In contrast, both genes significantly reduced the susceptibility to apoptosis by c-Myc elicited by serum withdrawal but not to apoptosis induced by thapsigarin (see Figure 3E). Taken together, these results supported the conclusion that Bin1 was necessary for c-Myc-induced death.

Although in previous studies we did not observe cytotoxic effects of Bin1 in untransformed cells (Sakamuro *et al.* 1996; Wechsler-Reya *et al.* 1998), since results from this study argued that Bin1 had a necessary role in apoptosis by c-Myc, we further investigated in CEFs whether Bin1 was sufficient to induce death by itself or to augment it in the presence of deregulated c-Myc. Using a recombinant retrovirus to deliver wild-type Bin1 we observed no augmentation of apoptosis when c-Myc was coexpressed, indicating that endogenous levels of Bin1 were not limiting. Furthermore, we observed that Bin1 was insufficient by itself to induce cell death in the absence of c-Myc overexpression (data not shown). These observations corroborated others indicating that overexpression of Bin1 is insufficient to induce apoptosis in the absence of c-Myc deregulation, including in primary rat embryo fibroblasts (REFs), C2C12 mouse myoblasts, IMR90 human diploid fibroblasts, or normal human melanocytes (Elliott *et al.* 1999; Ge *et al.* 1999; Sakamuro *et al.* 1996; Wechsler-Reya *et al.* 1998). Taken together, the results indicated that Bin1 had a selective and necessary role in apoptosis by c-Myc that was manifested only if c-Myc was deregulated. We concluded that Bin1 mediated apoptosis by c-Myc in a manner that was consistent with a role as a death adaptor protein.

Bin1 is required for c-Myc to induce p53-independent apoptosis in epithelial cells

We showed previously that deregulated c-Myc efficiently induces p53-independent apoptosis in baby rat kidney (BRK) epithelial cells (Sakamuro *et al.* 1995). We investigated a role for Bin1 in this process using LTR.1A, a BRK cell line developed in that study by concerted immortalization with human c-Myc and a temperature-sensitive dominant inhibitory p53 mutant. p53-independent apoptosis is induced efficiently by c-Myc in LTR.1A cells by serum deprivation at the nonpermissive temperature (38°C) for wild-type p53 function (Sakamuro *et al.* 1995). LTR.1A cells were infected by recombinant murine retroviruses expressing Bin1 AS, Bin1Δ4, or no insert, and the neomycin resistance cassette on each vector was selected for by culturing cells in G418. Drug-resistant cells were pooled and exogenous expression of Bin1 AS and Bin1Δ4 was confirmed by RT-PCR as before. No differences were observed in the number of colonies which emerged following infection nor in cell morphology, consistent with the likelihood that neither gene was growth inhibitory (data not shown). Effects on proliferation and apoptosis of BRK LTR.1A cells were assessed as before. Bin1 AS and Bin1Δ4 expression promoted the growth of LTR.1A cells relative to vector controls (see Figure 4A). The effect was similar to the trend noted in CEFs but stronger (compare Figures 1B and 3C), possibly reflecting the difference in the status of p53 in CEFs (wild-type) versus in LTR.1A cultured at 38°C (mutant). p53-independent apoptosis was induced by culturing the LTR.1A cell populations at 38°C in media containing 0.1% serum, which maintains the temperature-sensitive p53 mutant in its dominant inhibitory state (Sakamuro *et al.* 1995). Similar to the parental cell line, the vector control cells exhibited strong apoptotic death. In contrast, the cells expressing the Bin1 AS and Bin1Δ4 genes exhibited significant resistance to apoptosis (see Figures 4B and 4C). The effect of Bin1 AS and Bin1Δ4 was not due to a reduction of c-Myc levels in the BRK cells (data not shown), ruling out the trivial possibility that resistance was due to reduced levels of c-Myc that were sufficient to support immortalization but not apoptosis. We concluded that Bin1 was necessary for c-Myc to induce apoptosis in epithelial cells via a p53-independent mechanism(s).

Bin1 inhibition supports outgrowth of c-Myc-expressing cells under low serum conditions

c-Myc is sufficient to drive cell cycle progression in the absence of growth factors (Eilers *et al.* 1989). Therefore, if Bin1 is necessary for c-Myc to drive apoptosis but dispensable for it to promote cell proliferation, then inhibition of Bin1 should promote the outgrowth of c-Myc-expressing cells when they are deprived of growth factors (a situation which would normally lead to their apoptotic demise). To test this hypothesis we performed colony formation assays in Rat1A fibroblasts, which are quite sensitive to the cytotoxic effects of c-Myc. Rat1A cells were transfected with the strong human c-Myc vector LTR Hm and neomycin resistance gene-tagged vectors for Bin1 AS , Bin1Δ4, mutant p53, Bcl-2, or no insert. Rat1A fibroblasts have wild-type p53, which sensitizes them to apoptosis by c-Myc (Wagner *et al.* 1994), so coexpression-expression of mutant p53 was expected to blunt the cytotoxicity of c-Myc in these cells in a manner similar to Bcl-2 (Fanidi *et al.* 1992; Wagner *et al.* 1993). G418 selection was imposed in media containing 2% serum. This level of growth factors is insufficient to block apoptosis of rodent fibroblasts overexpressing c-Myc (Evan *et al.* 1992), so colonies would form only if cell growth could outpace cell death in the colony. After two weeks, G418-resistant colonies were stained and counted. As expected, cells transfected with LTR Hm and empty vector and cultured under these conditions formed significantly fewer colonies compared to the vector only control (see Figure 5A) The inhibitory effect of c-Myc on colony formation in 2% serum was relieved by cotransfection of Bcl-2 or also by mutant p53. Bcl-2 cooperates with c-Myc in Rat1 fibroblasts by blocking apoptosis (Fanidi *et al.* 1992; Wagner *et al.* 1993), validating this assay as a measurement of antiapoptotic potential. Notably, cotransfection of Bin1 AS or Bin1Δ4 also relieved c-Myc cytotoxicity and the relief was as potent as that provided by Bcl-2 (see Figure 5A). Experiments in which the ratio of c-Myc to Bin1 AS vector was varied in the transfected DNA demonstrated that the inhibitory effect was titratable (see Figure 5B). We noted that the effect on colony number by Bin1 AS or Bin1Δ4 was similar but that Bin1 AS had a more pronounced effect on colony size (see Figure 5C), indicating its action was slightly stronger. The results corroborated findings in CEFs and BRKs

which indicated that inhibition of Bin1 selectively suppressed apoptosis but not proliferation by c-Myc. We concluded that Bin1 mediated the cytotoxicity of c-Myc and thereby limited the ability of c-Myc to drive cell proliferation under low serum conditions.

Discussion

This study offers evidence of a selective and necessary role for the c-Myc binding adaptor protein Bin1 in the mechanism by which c-Myc activates apoptosis. In chick fibroblasts, where c-Myc is sufficient to drive proliferation and malignant transformation, as well as to activate apoptosis, Bin1 was shown to be necessary for the proapoptotic but not the transforming properties of c-Myc. The requirement for Bin1 in apoptosis by c-Myc was manifested in both chick and rodent fibroblasts, where p53 status is reported to be important (Hermeking and Eick 1994; Wagner *et al.* 1994), but also in epithelial cells, where it is not (Sakamuro *et al.* 1995). Notably, the c-Myc binding domain (MBD) of Bin1 was a potent and specific inhibitor of cell death. Since this domain is necessary and sufficient for interaction with c-Myc (Elliott *et al.* 1999; Sakamuro *et al.* 1996), its antiapoptotic action implied that interaction between Bin1 and c-Myc is necessary for death and that Bin1 is a specific death effector or coactivator which acts downstream or in parallel to c-Myc, respectively. Bin1 was not sufficient to induce death when overexpressed in the absence of deregulated c-Myc. Thus, Bin1 is not an 'executioner' but an adaptor protein that under certain circumstances can link c-Myc to a bona fide death pathway. In growing cells, c-Myc and Bin1 are coexpressed, colocalized, and can be coimmunoprecipitated (Elliott *et al.* 1999; Wechsler-Reya *et al.* 1997; Wechsler-Reya *et al.* 1998), other events or conditions must potentiate the death signal which Bin1 is needed to mediate. Since c-Myc and Bin1 are each phosphoproteins and c-Myc is known to be regulated by phosphorylation (Lutterbach and Hann 1994; Wechsler-Reya *et al.* 1997), it is tempting to speculate that the activity of each protein is regulated by kinases that are in turn regulated by survival factors. Pathways regulated by insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF) are intriguing in this regard since both factors have been

shown to be sufficient to suppress apoptosis by c-Myc in fibroblasts (Harrington *et al.* 1994). This study prompts investigation of the role of phosphorylation in regulating proapoptotic functions of Bin1.

We observed that Bin1 was necessary for apoptosis by c-Myc in BRK epithelial cells where p53 is dispensable. Thus, Bin1 may act in parallel or downstream of p53, providing a mechanism to explain how c-Myc can kill in certain cell types when p53 is abolished by mutation. The exact relationship between c-Myc and p53 in apoptosis is uncertain. While several studies performed in fibroblast and lymphocyte models suggest p53 is required (Hermeking and Eick 1994; Lotem and Sachs 1995; Wagner *et al.* 1994; Wang *et al.* 1993), and suggest a linkage by $p19^{ARF}$ (Zindy *et al.* 1998), other studies performed in quite similar models argue strongly against a role for p53 (Hsu *et al.* 1995; Lenahan and Ozer 1996). p53 is clearly not required in some cell types, such as BRK epithelial cells, where c-Myc can promote apoptosis by p53-dependent or p53-independent mechanisms (Sakamuro *et al.* 1995). Tissue-specific cooperation of p53 and c-Myc in apoptosis is suggested by the observation that c-Myc activation and p53 inactivation cooperate to promote thymic lymphoma but not mammary carcinoma (Elson *et al.* 1995). A recent study of the p53 modulator $p33^{ING1}$ in cells where apoptosis is induced by deregulated c-Myc is compatible with the notion that the death pathways activated by p53 and c-Myc are arranged in a parallel rather than strictly epistatic configuration (Helbing *et al.* 1997). One way to consider the relationship between p53 and c-Myc is to propose that each sensitize cells to apoptosis determined by each other or to other factors, such as, for example, DNA damage in the case of p53 (Lane 1992), or Fas or TNF in the case of c-Myc (Hueber *et al.* 1997; Klefstrom *et al.* 1997). In this scenario, Bin1 might mediate a death sensitization signal from c-Myc that could act independently but also cooperatively with p53 elevation. Indeed, a general role for Bin1 in sensitizing cells to apoptotic signals should be entertained, because the Bin1 gene appears to be a transcriptional target of the apoptosis-regulating factor NF-κB (Mao *et al.* 1999). It will be interesting to investigate links between Myc, Bin1, and p53 in BRK epithelial cells which exhibit two modes of apoptosis by c-Myc (Sakamuro *et al.* 1997),

and to determine whether Bin1 is necessary for Myc and/or p53 to sensitize cells to apoptosis by stimuli other than growth factor deprival.

Our findings support the 'dual signal' model which proposes that regulation of apoptosis is a physiological component of c-Myc action (Harrington *et al.* 1994; Harrington *et al.* 1994). Others have shown that dibutyryl cAMP can arrest cells containing deregulated c-Myc without affecting their sensitivity to apoptosis by growth factor deprival, consistent with the notion that different effectors of Myc mediate its different biological effects (Packham and Cleveland 1996). Thus, c-Myc may induce cell death via a novel effector pathway involving Bin1 and induce cell proliferation and transformation through other c-Myc binding proteins, such as, for example, the recently described ATM-related protein TRRAP, which is crucial for c-Myc-dependent cell transformation (McMahon *et al.* 1998). The 'dual signal' model shown in Figure 6 incorporates Bin1 into a p53-independent apoptosis pathway. As mentioned above, Bin1 is a phosphoprotein and therefore conceivably a target for regulation by growth factors that modulate apoptosis by c-Myc (Harrington *et al.* 1994). This model does not distinguish a specific role for Bin1 in cases where c-Myc is overexpressed rather than merely deregulated, an issue that may contribute to whether p53-dependent or p53-independent mechanisms for apoptosis may be favored in cells. However, at this time we do not favor such a distinction, because the potency of the apoptotic activity of Bin1 in tumor cells does not correlate with their degree of c-Myc overexpression (K. Ge, K. Elliott, and G.C.P., unpublished observations). Interestingly, recent experiments indicate that Bin1 induces tumor cell death by a caspase-independent mechanism associated with membrane blebbing (Elliott *et al.* 1999), reminiscent of the features of cell death induced by c-Myc in the presence of caspase inhibitors (McCarthy *et al.* 1997). Thus, Bin1 may address the gap in knowledge concerning how c-Myc triggers commitment to death independently of caspases and at a point before caspases come into play (McCarthy *et al.* 1997).

Although its exact function remains obscure, c-Myc has been implicated in transcriptional regulation and ~30 genes regulated by c-Myc have been identified (Dang 1999; Facchini and Penn 1998; Henriksson and Lüscher 1996; Prendergast 1997). However, we did not detect any changes in the expression of several target genes implicated in apoptosis by Myc, including ornithine decarboxylase, CDC25A, or Fas ligand (Galaktionov *et al.* 1996; Hueber *et al.* 1997; Packham and Cleveland 1994), associated with altered apoptotic responses in CEFs or BRKs (unpublished results). These observations are consistent with the lack of an unambiguous role for any of the c-Myc target genes identified to date in apoptosis (Dang 1999; Evan and Littlewood 1998). In transient assays, Bin1 can specifically suppress Myc transactivation (Elliott *et al.* 1999), so if this activity is physiologically germane, then other target genes may be important. Conversely, if the effects of Bin1 on transactivation are an epiphenomenon of protein-protein association as scored in transient assays, then Bin1 may have a signaling role that is independent of transactivation. The latter possibility needs to be entertained because not all biological actions of c-Myc can be ascribed strictly to gene transactivation (Gusse *et al.* 1989; Lemaitre *et al.* 1995; Li *et al.* 1994; Prendergast and Cole 1989; Yang *et al.* 1991) and Bin1 has features of a signaling protein (e.g. it has an SH3 domain). Moreover, c-Myc-induced death appears to be separable into "priming" and "initiation" steps, in which the former is associated with gene regulation but the latter is not, based on the ability of c-Myc to trigger cell death when protein synthesis is inhibited (Wagner *et al.* 1994). Identification of Bin1 effectors in cell death are needed to unravel its relationship with transcription by c-Myc, if any.

The effector functions of Bin1 are clearly of interest but currently undefined. Bin1 is in excess to Myc in cells and it has Myc-independent roles in cell regulation (Elliott *et al.* 1999). Thus, like most adaptor proteins, Bin1 probably participates in diverse interactions in the cell. Current studies of Bin1 support some role in coordinating cell fate decisions that are made when cells exit the cell cycle (e.g. arrest in G0, commit to differentiate, undergo apoptosis, etc.). For example, as shown above, if cells can not exit the cell cycle due to c-Myc deregulation, then Bin1 is necessary to

mediate an abortive apoptotic signal. Alternately, if c-Myc is downregulated appropriately and as a result cells can exit the cell cycle, then Bin1 promotes cell differentiation (Wechsler-Reya *et al.* 1998). Additional information indicates that the function of Bin1 is complex. Bin1 is subjected to complex patterns of alternate splicing, especially in neurons (Butler *et al.* 1997; Ramjaun and McPherson 1998; Ramjaun *et al.* 1997; Tsutsui *et al.* 1997; Wechsler-Reya *et al.* 1997), and it is localized to the cytosol as well as the nucleus in certain cells (Butler *et al.* 1997; Kadlec and Pendergast 1997; Wechsler-Reya *et al.* 1998). The terminal regions of Bin1 are structurally similar to amphiphysin, a neuron-specific protein and paraneoplastic autoimmune antigen in breast and lung cancer (David *et al.* 1994; Dropcho 1996), and to RVS167 and RVS161, two negative regulators of the cell cycle in yeast (Bauer *et al.* 1993; Crouzet *et al.* 1991). Amphiphysin and brain-specific splice forms of Bin1, also termed amphiphysin II or amphiphysin isoform, have been implicated in receptor-mediated endocytosis (David *et al.* 1996; Owen *et al.* 1998; Wigge *et al.* 1997). RVS167 and RVS161 have been implicated in endocytosis and karyogamy (Brizzio *et al.* 1998; Munn *et al.* 1995). Nonneuronal splice forms of Bin1 are unlikely to be involved in endocytosis, however, because only neuronal splice forms include exons which encode clathrin-binding determinants needed for localization to endocytotic vesicles (Ramjaun and McPherson 1998). It is tempting to speculate that the endocytosis connection in neurons reflects the link in those cells between survival and the achievement of a differentiated and synaptically active state associated with neurotransmitter release and hence membrane trafficking. Recently, the nuclear tyrosine kinase c-Abl was shown to associate with but not to phosphorylate Bin1 in cells (Kadlec and Pendergast 1997). Association with c-Abl is mediated by the SH3 domain in Bin1, which is dispensable for association with c-Myc (Elliott *et al.* 1999; Sakamuro *et al.* 1996). How Bin1 influences the complex actions of c-Abl in cell growth, differentiation, and apoptosis remains to be determined. However, in light of c-Abl interaction, it is interesting to note that a fraction of c-Abl in cells has been reported to be activated by localization to focal adhesions (Lewis *et al.* 1996; Taagepera *et al.* 1998), and that apoptosis by c-Myc is suppressed by signaling from integrins (Crouch *et al.* 1996), which localize to focal adhesions. Thus, there may be a link between apoptosis by c-Myc and Bin1, integrin signaling, and

activation of c-Abl. In future work, it will be important to determine whether any of the existing interactions mediate Bin1 actions in cell death or differentiation or are instead regulatory in nature.

Loss of Bin1 in cancer cells may promote c-Myc deregulation by eliminating an abortive apoptotic signaling mechanism that limits the consequences of inappropriate c-Myc expression. Loss or alteration of a proapoptotic adaptor such as Bin1 would achieve this end and leave proliferation intact. Apoptosis is crucial to stanch inappropriate cell proliferation but apoptotic mechanisms are progressively eliminated during neoplastic progression (Williams 1991). In certain cancers, such as prostate and breast cancer (Kyprianou *et al.* 1991; Kyprianou *et al.* 1990; McDonnell *et al.* 1992), there is evidence that loss of such mechanisms correlates with malignant conversion. p53 mutation is probably important but other yet undefined events also seem likely to be crucial. For example, as mentioned above, while p53 null mice are more susceptible to c-Myc-induced thymic lymphoma they have the same susceptible as wild-type mice to c-Myc-induced mammary carcinoma (Elson *et al.* 1995). Bin1 has features of a tumor suppressor that is epigenetically altered or eliminated in certain cancers, including prostate cancer, breast cancer, and melanoma (DuHadaway *et al.* 1999; Ge *et al.* 1999; Ge *et al.* 1999), where at later stages c-Myc is often overexpressed and associated with poor prognosis (Berns *et al.* 1992; Borg *et al.* 1992; Hehir *et al.* 1993; Jenkins *et al.* 1997). It will be important to investigate the effects of Bin1 loss in animal tumor models where its potential contribution to c-Myc deregulation and malignant development can be fully assessed.

Materials and Methods

Plasmid and retrovirus construction. The expression vectors used in this study have been described. pcDSRα is a strong mammalian vector which uses a hybrid SV40 early region/HTLV1 U5 LTR enhancer promoter to drive expression (a gift of A. Noda). MSCVneoEB is a murine MLV retroviral vector that includes a neomycin resistance gene cassette (Hawley *et al.* 1994). RCAS (BP) (A and B envelope subtypes) and RCOS (BP) (A envelope subtype) are related chicken retroviral vectors that are competent for replication (kindly provided by S. Hughes). RCAS (BP) contains the avian leukemia virus long terminal repeat (LTR) which is about ~10-fold more active than the RAV-0 LTR in RCOS (Petropoulos and Hughes 1991). A C-terminal fragment of murine Bin1 encompassing the Myc-binding domain (MBD) which was engineered for expression by the addition of a Kozak translation initiation sequence, termed ATG99, has been described previously (Sakamuro *et al.* 1996). ATG99 is sufficient for interaction with c-Myc and dominantly interferes with c-Myc-Bin1 interaction *in vivo* (Sakamuro *et al.* 1996). ATG99 was subcloned into RCAS (BP)-B to generate RCAS-MBD. The dominant inhibitory mutant Bin1Δ4 was generated by standard PCR methodology as part of a structure-function analysis to identify crucial regulatory regions of Bin1 (Elliott *et al.* 1999). Bin1Δ4 lacks aa 143-148 of full length human Bin1 (Sakamuro *et al.* 1996). Antisense Bin1 and Bin1Δ4 cDNAs were subcloned for mammalian cell expression into pcDSRα and MSCVneoEB, generating SRα-Bin1 AS and SRα-Bin1Δ4 or MSCV-Bin1 AS and MSCV-Bin1Δ4. For chick expression the same cDNAs were subcloned into RCAS (BP)-B, generating RCAS-Bin1 AS or RCAS-Bin1Δ4, via the adaptor cloning plasmid pCla12-Nco (Hughes *et al.* 1987). A human c-Myc cDNA derived from CMV Hm (Prendergast *et al.* 1991) was subcloned into RCAS (BP)-A or RCOS (BP)-A to generate RCAS-c-Myc or RCOS-c-Myc. An RCAS-Bcl-2 vector has been described (Givol *et al.* 1994). For rat embryo fibroblast (REF) transformation experiments, Bin1Δ4 was subcloned into the cytomegalovirus (CMV) enhancer/promoter-driven vector pcDNA3 (Invitrogen), generating CMV-Bin1Δ4, to permit comparison of activity with CMV-Bin1 (Sakamuro *et al.* 1996). Similar CMV vectors for the murine temperature-sensitive dominant inhibitory mutant

p53 and human Bcl-2 have been described (Sakamuro *et al.* 1995). The activated H-ras vector pT22 and the human c-Myc vector LTR Hm used for REF focus assays also have been described (Kelekar and Cole 1986; Land *et al.* 1983).

Tissue culture and cell line generation. All cells were cultured in Dulbecco's modified Eagle media (DMEM) containing 10% fetal calf serum (Life Technologies) and penicillin/streptomycin unless otherwise indicated. Primary chick embryo fibroblasts (CEFs) were cultured from 10 day old chick embryos (EV-0 strain) by standard technique and grown in DMEM supplemented with 8% fetal calf serum, 2% chicken serum, 10% Tryptose Phosphate Broth, and antibiotics. Low passage CEFs seeded into 100 mm dishes were transfected with 5 μg each of the desired recombinant retroviral vector DNAs by a standard calcium phosphate coprecipitation method (Chen and Okayama 1987). Cells were cultured 4-7 days and passaged every other day to promote propagation of the recombinant viruses. Complete propagation was confirmed by immunofluorescence staining with an antibody to the viral core protein p27 (SPAFAS, Inc.) and culture supernatant was harvested and stored at -80°C. Viral titers achieved in this manner were typically ~10^6/ml. To generate CEF populations carrying two transgenes, cells were transfected with A subtype viral vectors, cultured 4 days, and then infected with B subtype viruses generated as above. Growth curves were performed by seeding 2 x 10^5 cells into 6 cm dishes and counting viable cells at various times later. Soft agar culture to assay anchorage-independent growth was performed by seeding 2.5 x 10^4 cells per well in triplicate into 6 well dishes, as described previously (Press *et al.* 1992), except that concanavalin A was omitted. Colonies were documented by photography at 40x magnification using an Olympus inverted microscope with a 35 mm camera attachment.

The BRK myc/p53ts cell line LTR.1A has been described previously (Sakamuro *et al.* 1995). LTR.1A cells were infected with MSCV retroviruses harvested 48 hr after transient transfection of 293-BOSC cells as described (Pear *et al.* 1993). Cells stably integrating the vector were selected in growth media containing 0.5 mg/ml G418 and pooled. Primary rat embryo fibroblasts (REFs)

(Whittaker Bioproducts) were cultured and transfected as described (Prendergast *et al.* 1992; Prendergast *et al.* 1991). Transformed foci were scored 12-16 days posttransfection. Rat1A cells (a gift of N. Kohl) were used for colony formation assays as follows. ~3 x 10^5 cells were seeded per well in 6-well dishes and transfected the next day by a standard calcium phosphate coprecipitation method (Chen and Okayama 1987) with 6 μg LTR Hm and 6 μg of empty vector, pSRα-Bin1 AS, pSRα-Bin1Δ4, CMV-p53ts, or CMV-Bcl-2 (Sakamuro *et al.* 1995). Cells were trypsinized 48 hr after transfection and seeded at a 1:20 ratio into 10 cm dishes containing DMEM media supplemented with 2% fetal calf serum and 0.6 mg/mL G418 (Life Technologies). G418-resistant colonies were scored by crystal violet staining 14 days later.

RNA analysis. The human Bin1 specific primers Δ4-sense (5'-AGT TCC CCG ACA TCA AGT CAC GCA-3') and Δ4-antisense (5'-CTT GGC AAT TTT GGC TTC ATC C-3'), which span the 18 nt deletion in Bin1Δ4, were used to document exogenous expression of Bin1Δ4 message in cells. Reverse Transcriptase (RT)-PCR analysis was performed as follows. Two μg total cytoplasmic RNA was mixed with 50 pmol of each primer, heated to 70°C for 5 min, and cooled rapidly on wet ice. RT reaction was performed in 30 μl as suggested by the Mo-MLV RT vendor (Life Technologies). Ten percent of the reaction product was used as a template for 30 cycles of PCR (denaturation 30s at 94 °C/annealing 45 s at 55 °C/polymerization 60s at 72 °C). Ten percent of the PCR product was examined by agarose gel electrophoresis and photographed. For Northern analysis, 20 μg total cytoplasmic RNA per lane was analyzed essentially as described (Prendergast and Cole 1989). Blots were probed with cDNAs for murine ODC (a gift of J. Cleveland), murine CDC25A (a gift of D. Beach), murine Fas and Fas ligand (gifts of S. Nagata), or p16INK4-exon 1β specific for $p19^{ARF}$ (a gift of T. Kamijo).

Protein analysis. For Western blotting, cell lysates was prepared in NP40 buffer (Bin1 and Bcl-2) or RIPA buffer (c-Myc) using standard protocols (Harlow and Lane 1988). For c-Myc analysis, 1.5 mg cell lysate was subjected to immunoprecipitation with 1 μg anti-c-Myc antibody

SC-42 (Santa Cruz Biotechnology) and 25 µl protein G-agarose (Life Technologies) and immunoprecipitates were washed 3 times with RIPA buffer before fractionation by non-reducing SDS-PAGE. For Bin1 and Bcl-2 analysis, 50 µg cell lysate was fractionated directly by reducing SDS-PAGE. Gels were electrophoretically transferred to ECL membrane (Amersham) or Immobilon-P (Millipore) using standard methods (Harlow and Lane 1988). Blots were blocked in 3% skim milk and probed with the anti-Bin1 monoclonal antibody 99D (Wechsler-Reya *et al.* 1997), anti-c-Myc antibody 9E10 (Evan *et al.* 1985), or anti-Bcl-2 antibody #124 (DAKO). Antibodies were diluted 1:50 in PBS with 2.5% skim milk and 0.1% Triton X-100 and incubated with the membrane 12 hr at 4°C. Blots were washed and incubated 1 hr in the same buffer with secondary goat anti-mouse antibodies conjugated to horseradish peroxidase (BMB) and developed using a chemiluminescence kit using the protocol suggested by the vendor (Pierce). Indirect immunofluorescence of BRK cells and CEFs was performed with anti-Bin1 99D as described (Prendergast and Ziff 1991; Sakamuro *et al.* 1996).

Apoptosis assays. ~10^6 CEFs expressing the transgenes indicated were seeded overnight into 60 mm dishes in complete growth media for apoptosis assays. Recombinant retrovirus-infected BRK/myc/p53ts cells were grown to ~70% confluence in 100 mm dishes. At the start of the experiment, cells were washed twice with PBS and then placed in DMEM containing either 10%, 0.1%, or 0.05% fetal calf serum as indicated. Thapsigarin (Calbiochem) was added to complete growth media where indicated to a final concentration of 100 nM as a means to induce apoptosis by a c-Myc-independent mechanism. At the end of the incubation period, cells were trypsinized, washed once with PBS, fixed and stained with propidium iodide, and processed for flow cytometry (Givol *et al.* 1994; Sakamuro *et al.* 1995; Sakamuro *et al.* 1997). Alternately, cells were harvested, stained with acridine orange (Sigma), and subjected to fluorescence microscopy to monitor for chromatin condensation. Staining with acridine orange was performed by dissolving 2.5 mg in 50 mL of PBS at room temperature, mixing 2 µl with 10 µl of cell suspension on a slide glass, and covering with melted paraffin wax.

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Figure Legends

Figure 1. MBD overexpression does not inhibit proliferation or cell transformation by c-Myc. **(A).** Transgene expression. Cell extracts were prepared from CEFs infected with RCOS (A) vector or RCOS-c-myc plus RCAS (B) vector, RCAS-MBD, or RCAS-Bcl-2 and subjected to Western analysis with anti-c-Myc, anti-Bin1, or anti-Bcl2 antibodies. **(B.)** Anchorage-dependent growth. $2x10^5$ CEFs expressing the genes indicated were seeded into 60 mm dishes on day 0. Viable cell counts were determined at the times indicated and cell number was graphed as a function of time. **(C.)** Anchorage-independent growth. 2.5×10^4 CEFs were seeded into semisolid growth media in 6 well dishes as described in the Materials and Methods. Representative fields were documented by photomicrography at 40x magnification 10 days later.

Figure 2. MBD overexpression inhibits apoptosis by c-Myc. **(A.)** Apoptosis following serum deprival. CEFs expressing the transgenes indicated were incubated in growth media or in DMEM containing 0.05% fetal calf serum for 28 hr, trypsinized, fixed and stained with propidium iodide, and subjected to flow cytometry (Sakamuro *et al.* 1995; Sakamuro *et al.* 1997). The proportion of cells in each population exhibiting sub-G1 phase DNA is displayed on the X-axis. The results of three trials are shown **(B.)** Apoptosis following thapsigarin treatment. CEFs expressing the transgenes indicated were incubated in growth media containing 100 nM thapsigarin for 24 hr and processed as above for flow cytometry. The proportion of cells in each population exhibiting sub-G1 phase DNA is displayed on the X-axis. Cells also exhibited morphological features of apoptosis and chromatin collapse (data not shown). The results of three trials are shown.

Figure 3. Inhibition of c-Myc-induced apoptosis, but not transformation or proliferation, by antisense Bin1 or the dominant inhibitory effector mutant Bin1Δ4 . **(A.)** Bin1Δ4 is an effector mutant with dominant inhibitory activity. Bin1Δ4 lacks the perfectly conserved aa 143-148 within the BAR-C region of Bin1 which is crucial in addition to the MBD to inhibit c-Myc transforming

activity (Elliott *et al.* 1999). REF cotransformation assays were performed using c-Myc and oncogenic H-Ras vectors LTR Hm and pT22 as described previously (Prendergast *et al.* 1992). Briefly, cells were transfected with 5 μg each oncogene plus 5 μg of CMV vector and CMV-Bin1, CMV-Bin1Δ4, or CMV vector, or plus 5 μg each of CMV-Bin1 and CMV-Bin1Δ4. Foci were scored 2 weeks later and plotted as proportion of the foci scored in the presence of CMV vector alone. The results of 5 trials are shown. Coexpression of Bin1Δ4 and Bin1 in the foci pooled from one trial in which both genes were transfected was confirmed by RT-PCR (as described in the Materials and Methods), demonstrating that Bin1 could be expressed in cells transformed by c-Myc if Bin1Δ4 was coexpressed. **(B).** Transgene expression in CEFs. Bin1 activity is fouled by the addition of either C- or N-epitope tags (Elliott *et al.* 1999; Sakamuro *et al.* 1996) so expression of the untagged Bin1Δ4 gene was confirmed by RT-PCR analysis of total cytoplasmic RNA isolated from each cell population as described in the Materials and Methods. Primers were specific for human Bin1 and do not crosshybridize to chicken Bin1 sequences. CMV-Bin1 and CMV-Bin1Δ4 were used as positive control templates. Western analysis confirmed expression of Bin1Δ4 protein and indicated truncation to two ~40 kD fragments during preparation of cell lysates. Immunoprecipitation of endogenous chicken Bin1 from CEFs that were infected with Bin1 AS retrovirus and metabolically labeled with ^{35}S-methionine indicated a ~2-fold reduction in protein levels, a degree of suppression that was similar to that associated with biological effect by the same gene on Bin1 function in mouse C2C12 cells (Wechsler-Reya *et al.* 1998). **(C.)** Anchorage-dependent growth. $2x10^5$ CEFs expressing the genes indicated were seeded into 60 mm dishes on day 0. Viable cell counts were determined at the times indicated and cell number was graphed as a function of time. **(D.)** Anchorage-independent growth. 2.5×10^4 CEFs expressing the transgenes indicated were seeded into semisolid growth media in 6 well dishes as described in the Materials and Methods. Representative fields were documented by photomicrography at 40x magnification 10 days later. **(E.)** Apoptosis following serum deprivation or thapsigarin treatment. CEFs expressing the transgenes indicated were incubated ~22 hr in growth media, DMEM containing 0.1% fetal calf serum, or growth media containing 100 nM thapsigarin. At the end of this period cells were

harvested, fixed and stained with acridine orange or propidium iodide, and subjected to flow cytometry or to fluorescence microscopy (Sakamuro *et al.* 1995; Sakamuro *et al.* 1997). The graph shows the proportion of cells in each population which exhibited apoptosis and chromatin condensation. Relative cell death as measured by the proportion of sub-G1 phase cells exhibited a slightly more pronounced suppression by Bin1 AS and Bin1Δ4 than that determined by the chromatin condensation method shown (data not shown). The results of four trials are shown.

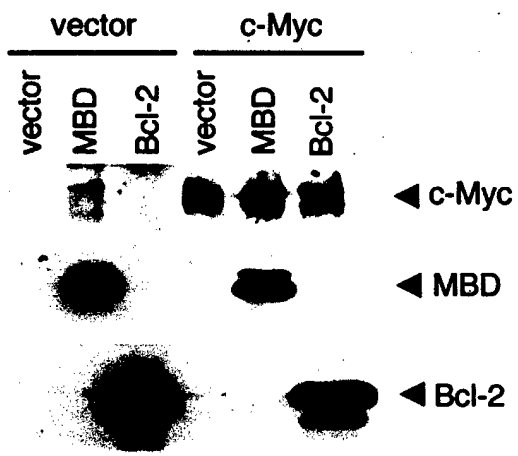
Figure 4. Bin1 is necessary for p53-independent apoptosis by c-Myc in BRK epithelial cells. **(A.)** Growth curve. 2 x 10^5 BRK LTR.1A cells expressing the genes indicated were seeded into 100 mm dishes on day 0. Viable cell counts were determined at the times indicated and cell number was graphed as a function of time. **(B.)** Response to serum deprival in the presence of p53 mutant. BRK LTR.1A cells were subjected to serum deprival as described previously (Sakamuro *et al.* 1995) and processed for flow cytometry. The relative proportion of cells exhibiting sub-G1 phase DNA is depicted on the X-axis of the graph. The results of three trials are shown. Similar suppression by Bin1 AS and Bin1Δ4 was observed in the BRK myc/p53ts cell line LTR.8C (Sakamuro *et al.* 1995) (data not shown). **(C.)** Photomicrographs of BRK LTR.1A at end of period of serum deprival.

Figure 5. Inhibition of Bin1 abolishes the cytotoxicity of c-Myc and promotes its ability to drive proliferation under low serum conditions. **(A.).** Relief of c-Myc cytotoxicity. Rat1A fibroblasts were transfected with 20 μg of LTR Hm or empty vector (to control for the promoter in LTR Hm) plus 5 μg of CMV vectors for the genes indicated. The CMV vector used, pcDNA3-neo, contains a neomycin-resistance cassette to permit G618 selection. G418-resistant colonies were scored 12-14 days after transfection. The results of three trials are shown. **(B.)** Titration of c-Myc cytotoxicity by Bin1 AS. Rat1A fibroblasts were transfected with 5 μg CMV-Bin1 AS or empty CMV vector (pcDNA3-neo) plus the amount of LTR Hm shown. Empty vector for the latter was used to control for the promoter in LTR Hm and to maintain plasmid amount to 20 μg in each transfection. G418-resistant colonies were scored as before. The results of three trials are shown.

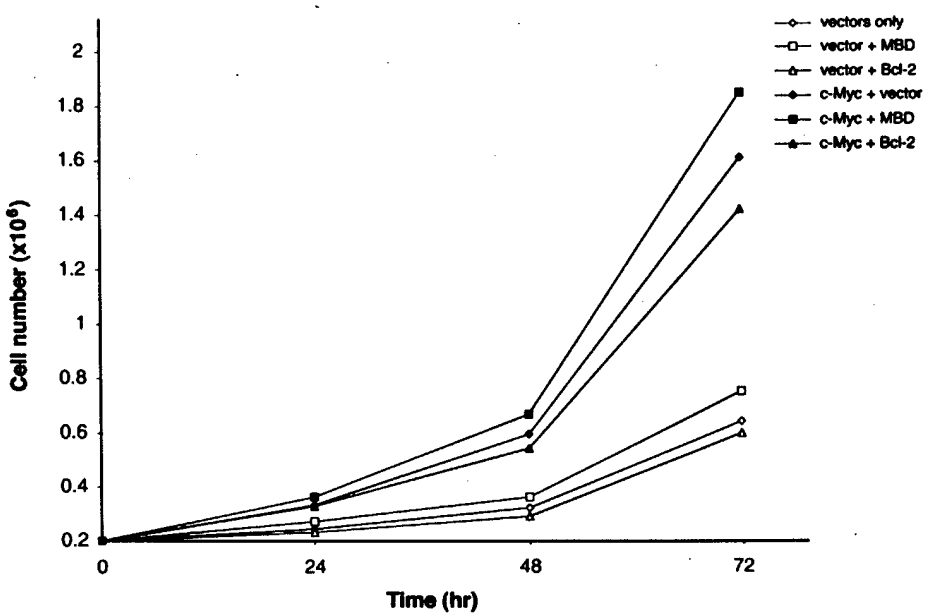
(C.) Representative dishes from one experiment are shown illustrating relief by Bin1 AS and Bin1Δ4 and slight difference on colony size.

Figure 6. Model. In the 'dual signal' model for Myc function, Bin1 is proposed to have a specific adaptor role in mediating a death or death sensitization signal from c-Myc. This signal is p53-independent so p53 may be involved in parallel or upstream of Bin1. Growth factors that suppress c-Myc-induced apoptosis may target Bin1 for inactivation, perhaps by phosphorylation. Bin1 is dispensable for c-Myc to drive proliferation or transformation, which may be mediated by other proteins that interact with the c-Myc N-terminus, such as TRRAP (McMahon *et al.* 1998).

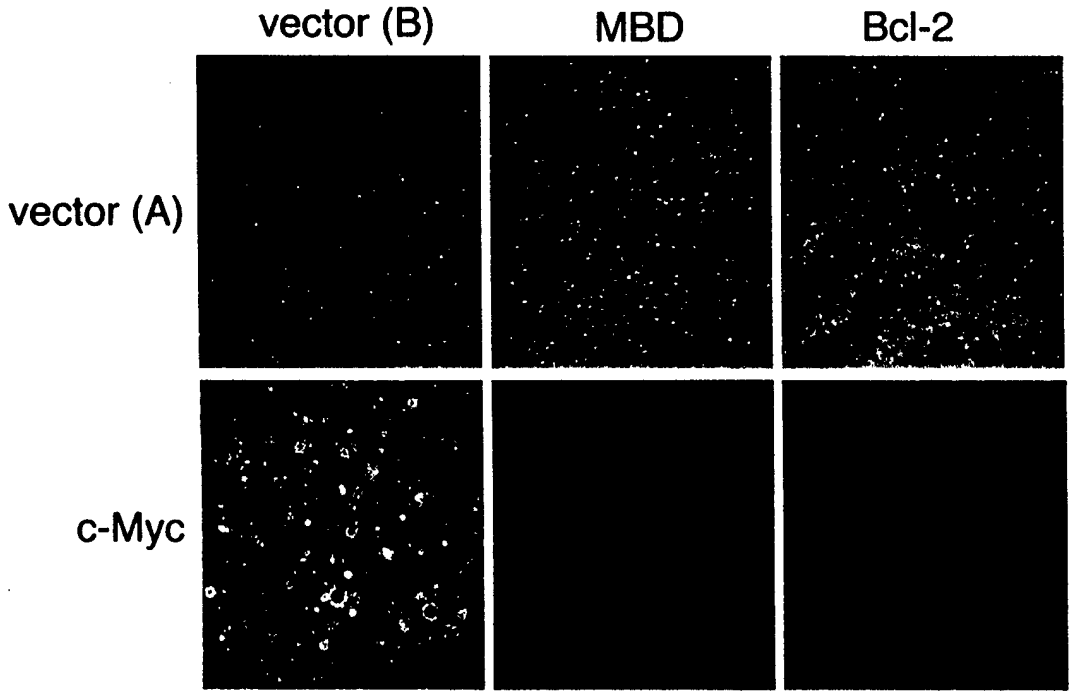
A)

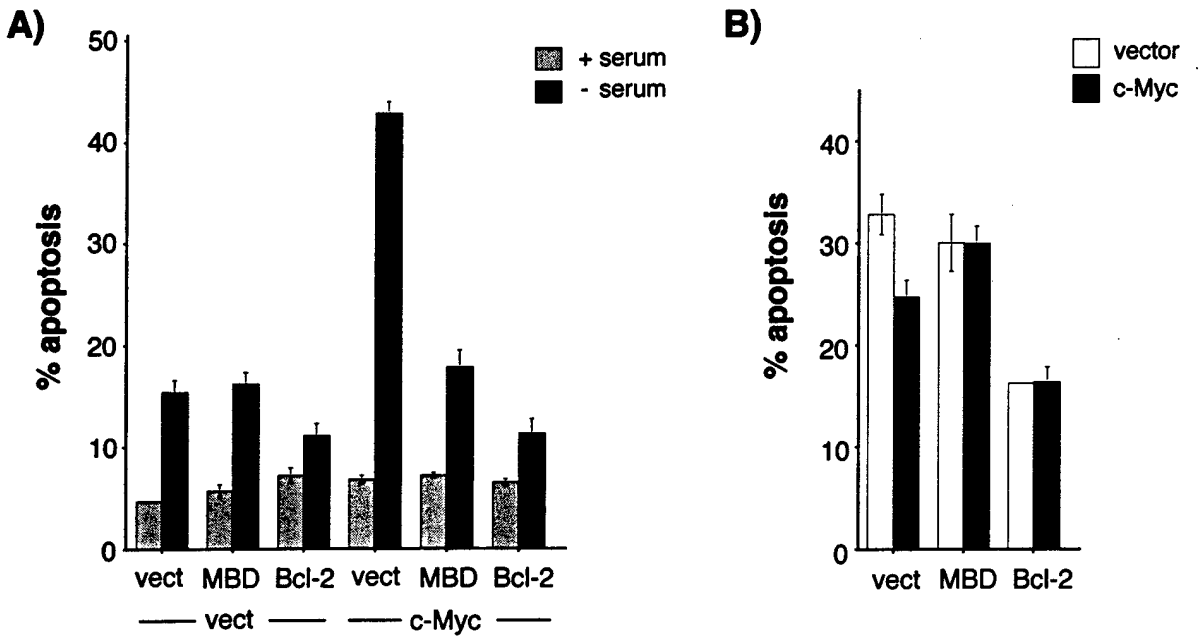

vector
c-Myc
vector
MBD
Bcl-2
vector
MBD
Bcl-2
c-Myc
MBD
Bcl-2

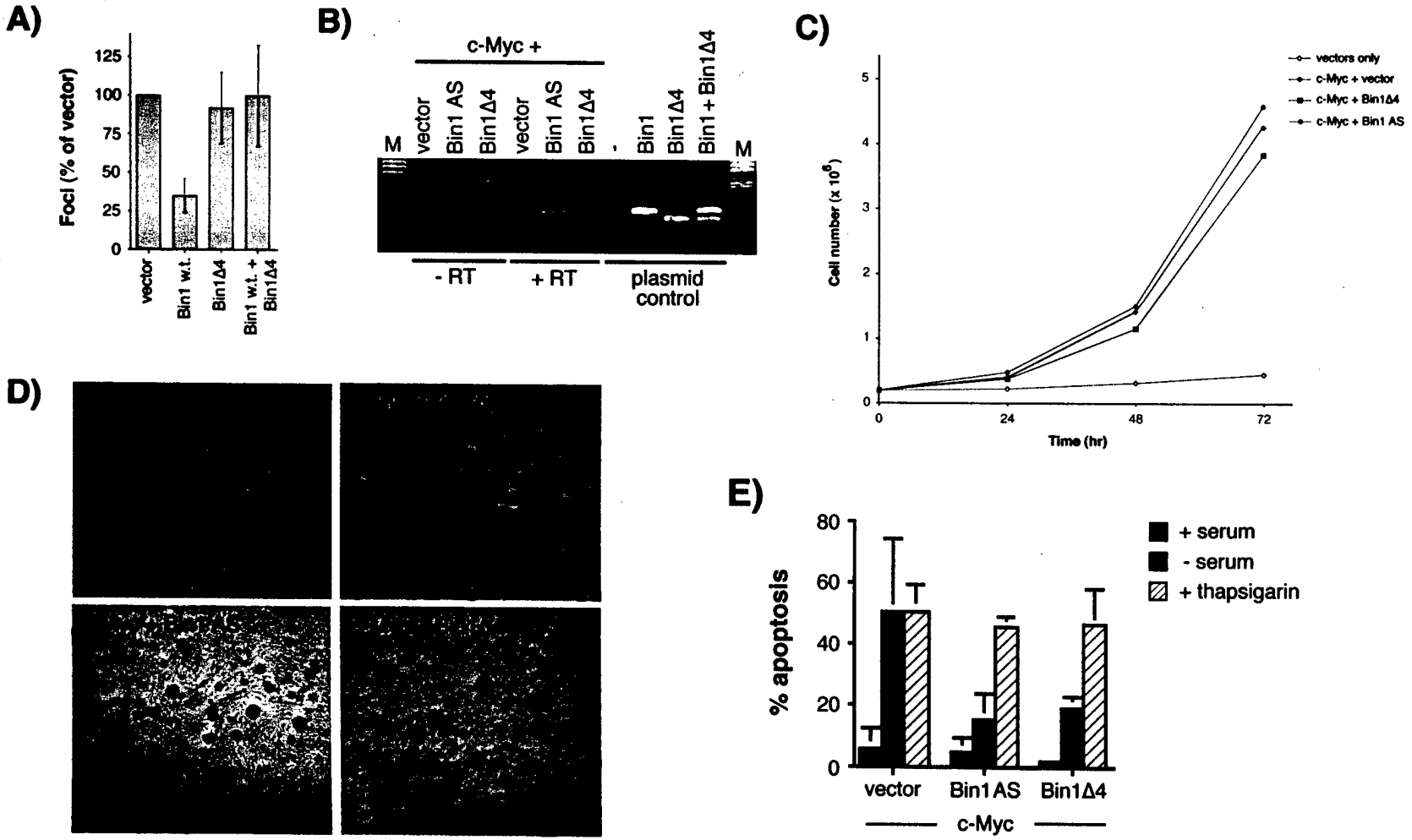
B)


vectors only
vector + MBD
vector + Bcl-2
c-Myc + vector
c-Myc + MBD
c-Myc + Bcl-2
Cell number (x10⁶)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24
48
72
Time (hr)

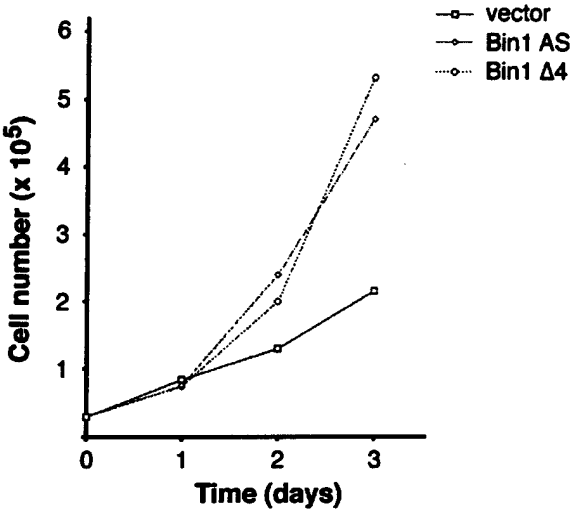
C)


vector (B)
MBD
Bcl-2
vector (A)
c-Myc

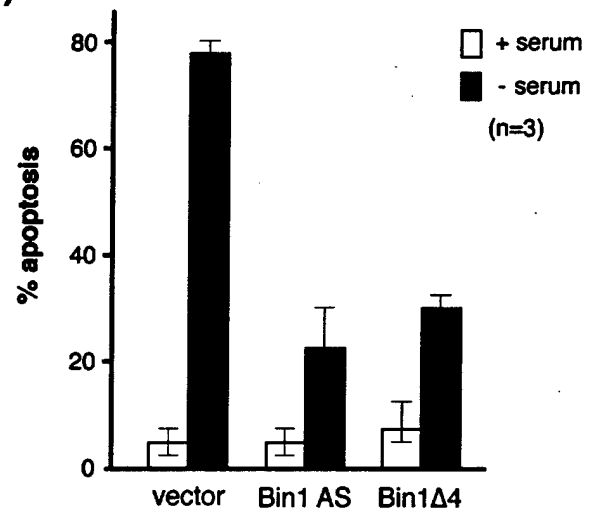

A)
% apoptosis
50
40
30
20
10
0
+ serum
- serum
vect
MBD
Bcl-2
vect
MBD
Bcl-2
vect
c-Myc
B)
vector
c-Myc
% apoptosis
40
30
20
10
0
vect
MBD
Bcl-2


A)
Foci (% of vector)
125
100
75
50
25
0
vector
Bin1 w.t.
Bin1Δ4
Bin1 w.t. + Bin1Δ4
B)
c-Myc +
M
vector
Bin1 AS
Bin1Δ4
vector
Bin1 AS
Bin1Δ4
-
Bin1
Bin1Δ4
Bin1 + Bin1Δ4
M
- RT
+ RT
plasmid control
C)
vectors only
c-Myc + vector
c-Myc + Bin1Δ4
c-Myc + Bin1 AS
Cell number (x 10^6)
5
4
3
2
1
0
0
24
48
72
Time (hr)
D)
E)
% apoptosis
80
60
40
20
0
+ serum
- serum
+ thapsigarin
vector
Bin1AS
Bin1Δ4
c-Myc

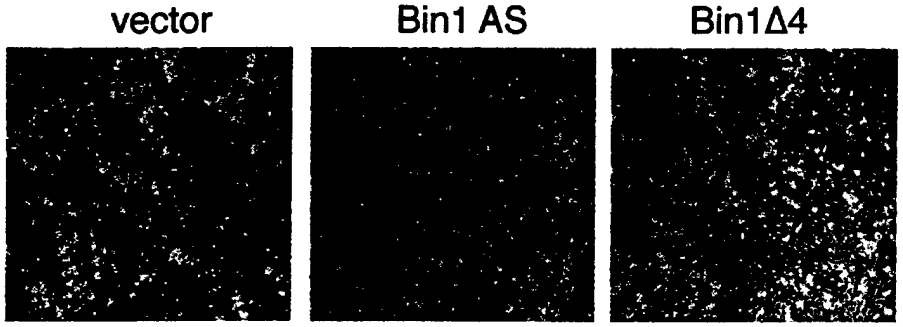
A)


vector
Bin1 AS
Bin1 Δ4
Cell number (x 10^5)
Time (days)
0
1
2
3
1
2
3
4
5
6

B)


+ serum
- serum
(n=3)
% apoptosis
0
20
40
60
80
vector
Bin1 AS
Bin1Δ4

C)


vector
Bin1 AS
Bin1Δ4

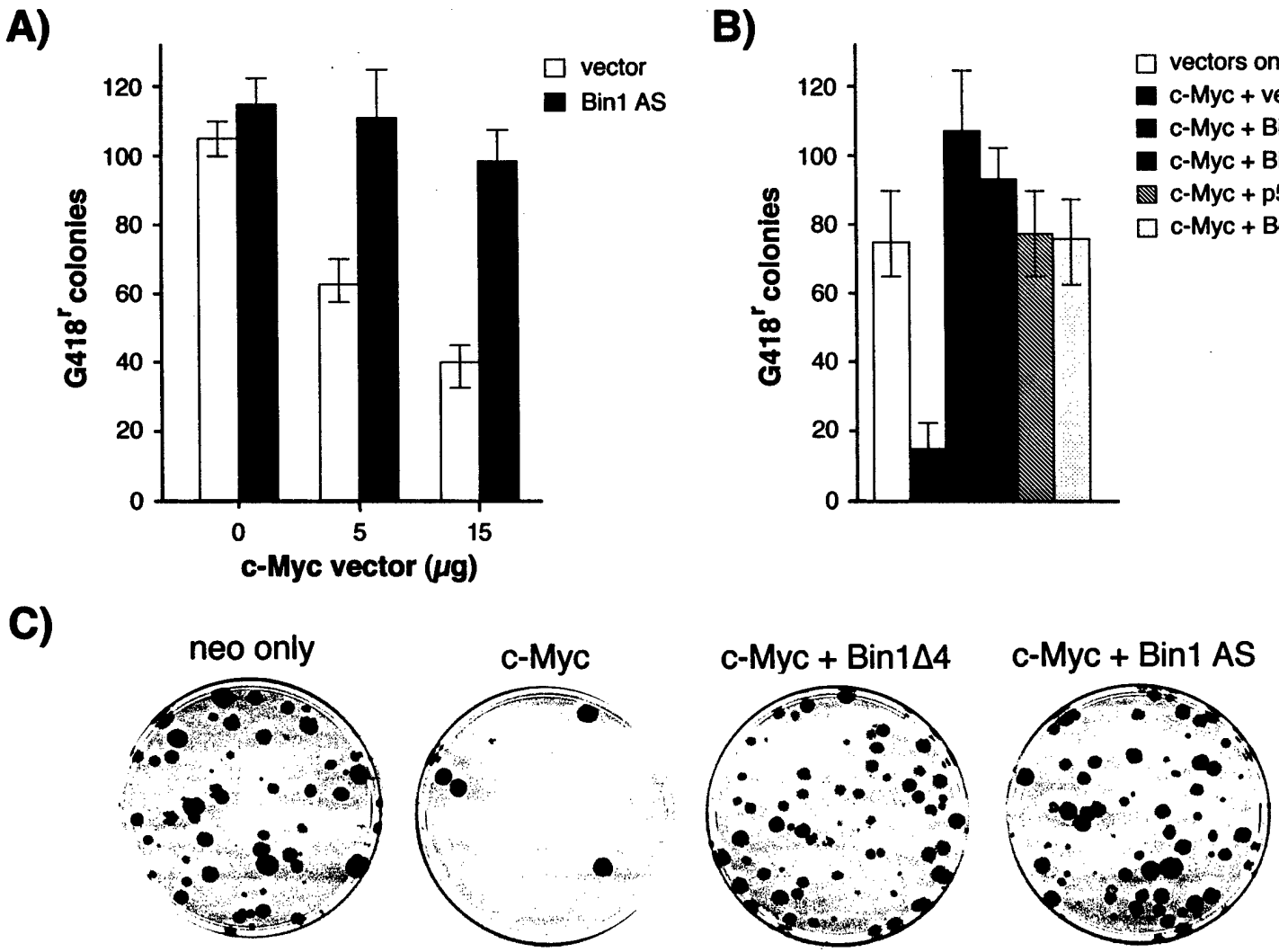
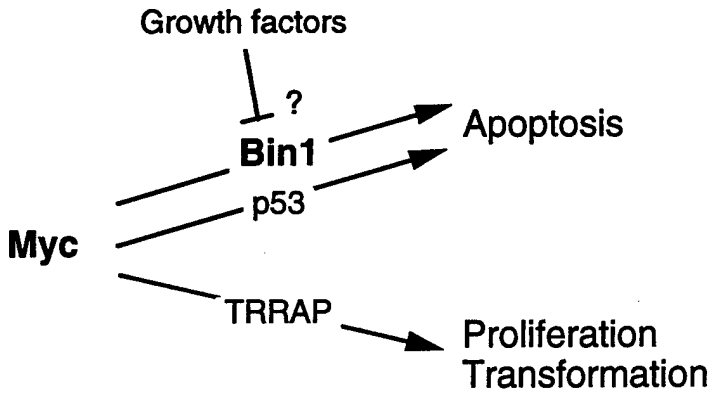

A)
G418r colonies
120
100
80
60
40
20
0
0
5
15
c-Myc vector (μg)
vector
Bin1 AS
B)
G418r colonies
120
100
80
60
40
20
0
vectors only
c-Myc + vector
c-Myc + Bin1 AS
c-Myc + Bin1Δ4
c-Myc + p53ts
c-Myc + Bcl-2
C)
neo only
c-Myc
c-Myc + Bin1Δ4
c-Myc + Bin1 AS

Fig. 6


Growth factors
?
Bin1
Apoptosis
p53
Myc
TRRAP
Proliferation
Transformation

Caspase-independent apoptosis is induced in tumor cells by the c-Myc-interacting adaptor protein Bin1

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Abstract

Caspase activation is associated with but dispensable for apoptosis by c-Myc. The c-Myc-interacting adaptor protein Bin1 was implicated recently as a necessary mediator of apoptosis by c-Myc. In this study, we report that when reintroduced into human tumor cells containing deregulated c-Myc Bin1 induces apoptosis by a caspase-independent mechanism. The features induced by Bin1 resembled those induced by c-Myc in the presence of caspase inhibitors. Death occured in all phases of the cell cycle and was characterized by membrane blebbing, cell shrinkage, substratum detachment, positive TUNEL reaction, and the appearance of sub-G1 phase DNA fragments by flow cytometry. p53 and Rb were dispensable because SAOS-2 osteosarcoma cells lacking these genes were sensitive to Bin1. In contrast, human diploid IMR90 fibroblasts expressing normal Bin1 were not susceptible. Apoptosis was associated with nuclear shrinkage but not with caspase activation, chromatin collapse, or nucleosomal DNA degradation. Consistent with the lack of a caspase requirement, caspase inhibitors did not block Bin1-induced cell death and in fact slightly potentiated it. These findings establish a proapoptotic and caspase-independent function for Bin1 which is missing or dysfunctional in cancer cells. We propose a model in which Bin1 mediates a c-Myc death signal that is coordinated with but separate from caspase activation.

Aberrant splicing and loss of antiproliferative activity of Bin1 in melanoma

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Abstract

The events that underlie the development of sporadic malignant melanoma are poorly understood. In this study, we identify the c-Myc-interacting tumor suppressor Bin1 as a target for epigenetic alteration in melanoma. Aberrant splicing of this adaptor protein was observed frequently in vertical growth phase and metastatic melanoma cells. In particular, aberrant splicing of the brain-specific exon 12A of the Bin1 gene was pinpointed as a common and biologically significant event. Inclusion of 12A-encoded sequences in Bin1 was sufficient to eliminate its ability to suppress malignant cell transformation by c-Myc or adenovirus E1A. In addition, melanoma cells expressing Bin1+12A isoforms were distinguished by their selective susceptibility to apoptosis induced by a recombinant Bin1 adenovirus. Our findings illustrate a novel epigenetic mechanism for alteration of Bin1 in cancer cells and suggest that loss of its antioncogenic activity may contribute to the melanoma development.

Alteration and loss of the tumor suppressor Bin1 in prostate carcinoma

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Abstract

The events leading to the development of prostate cancer remain to be fully defined. c-Myc amplification occurs frequently in late stage prostate cancer so events which contribute to deregulation of c-Myc may be important in this malignancy. Bin1 is a c-Myc-interacting adaptor protein that has features of a tumor suppressor. We examined prostate tumors for alteration or loss of Bin1 because the gene encoding it maps to the mid-chromosome 2q region which is deleted frequently in tumors with metastatic potential. Loss of heterozygosity (LOH) at the Bin1 locus was demonstrated in 6/15 (40%) informative cases of prostate carcinoma and in 5/6 of these cases alteration of the remaining allele was documented. In contrast, no LOH was observed in a panel of 18 cases of bladder carcinoma. Loss of expression of Bin1 occured in metastatic tumors and in the metastasis-derived androgen-independent cell lines PC3 and DU145. These deficits were significant because ectopic Bin1 inhibited the growth of DU145 and PC3 but not androgen-dependent LNCaP cells which retained normal endogenous Bin1. Our findings suggest that alterations of Bin1 may contribute to c-Myc deregulation and to prostate cancer progression.